REMARKS

Claims 22-43 were previously pending in this application. Claims 22, 32, 36 and 38 have been amended to delete IL-5 from the list of cytokines without prejudice to its subsequent presentation in a continuing application. As a result claims 22-43 are pending for examination with claims 22, 32, 36, and 38 being independent claims. No new matter has been added.

Rejections Under 35 U.S.C. §112

The Examiner has withdrawn the rejection of claims 22-31 under 35 U.S.C. §112 first paragraph in view of Applicants arguments filed April 8, 2003, Paper No. 11. The Examiner has maintained in part the rejection of claims 22-43 under U.S.C. §112, first paragraph, allegedly because the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with the claims.

The Examiner accepted Applicants' argument that the specification provides guidance with respect to the administration of the compounds of the invention to different subjects and routes of administration. The Examiner also accepted Applicants' argument that methods for delivering cytokines and CpG oligonucleotides to subjects have been described in many publications and patents. In addition, the Examiner accepted Applicants' argument that "the statement that targeting nucleic acids, like CpG, fall into the broad area known as gene therapy methods, is not scientifically correct."

However, the Examiner maintained the rejection under 35 U.S.C. §112 first paragraph in view of Broide et al. (Journal of Immunology, 1998 161, 7054-7062). The Examiner stated that Broide et al. disclose "that an immunostimulatory DNA containing a CpG motif significantly inhibited Th2 cytokines, IL-5 and IL-3 in mice." The Examiner alleged that the Broide et al. disclosure is inconsistent with the teachings of the present application. Applicants respectfully disagree. The teachings of Broide et al. relate to the administration of an immunostimulatory DNA ***alone***. In contrast, the present claims recite administering an "immunostimulatory CpG oligonucleotide" ***and*** "an immunopotentiating cytokine selected from the group consisting of IL-3 and IL-12." Broide et al. fail to disclose any combination of immunostimulatory DNA and cytokine. As such, Applicants submit that Broide et al. is not relevant to the evaluation of the present claims under 35 U.S.C. §112.

Applicants submit that the specification explicitly teaches that a combination of immunostimulatory oligonucleotide and a cytokine such as IL-3 or IL-12 has a synergistic effect on an immune response such as a "synergistic antigen specific immune response" in claim 22, "synergistically activating a dendritic cell" in claims 32 and 36, or "synergistically increasing survival time" in claim 38. See, for example, page 8, lines 9-12, page 38, paragraph 3, and page 8, paragraph 2. The specification further exemplifies a synergistic immune response in Example 9. Applicants submit that the disclosure of Broide et al. would not cause one of skill in the art to doubt these teachings in the present specification.

Furthermore, Applicants submit herewith a Declaration of Dr. Arthur Krieg, M.D., Senior Vice President for R&D and Chief Scientific Officer for Coley Pharmaceutical Group, and co-inventor on the instant application. In the Declaration, Dr. Arthur Krieg describes the results of several experiments that demonstrate the successful synergistic stimulation of an immune cell response in the presence of a CpG oligonucleotide in combination with either IL-3 or IL-12. The Declaration of Dr Arthur Krieg demonstrates that a combination of CpG ODN 2006 (SEQ ID NO:90, page 54 of the specification) and IL-3 synergistically produces an increase in IFNα (Exhibit 1). A synergistic increase in IFNγ is also demonstrated using a combination of either CpG ODN 2006 and IL-12, or CpG ODN 2395 (the sequence of ODN 2395 falls within the scope of the generic CpG oligonucleotide defined in the specification) and IL-12, or CpG ODN 1982 (SEQ ID NO:85, page 54 of the specification) and IL-12 (Exhibit 2).

In the instant specification, Applicants have shown that the combination of GM-CSF and CpG phosphorothioated ODN 2006 shows synergy for increasing the expression of costimulatory molecues CD86 and CD 40 on dendritic cells (Figure 9). Applicants have now presented evidence in the Declaration of Dr. Arthur Krieg, that the combination of CpG DNA ODN 2006 with cytokine IL-12, or CpG DNA ODN 2395 with IL-12, or CpG DNA ODN 1982 with IL-12, shows synergy for the increased expression of costimulatory molecules CD69 on human NK cells (Exhibit 3). Applicants submit that these experiments show that the teachings of the specification can be implemented without undue experimentation to obtain a synergistic response to a combination of an immunostimulatory CpG oligonucleotide and IL-3 or IL-12.

Therefore, Applicants believe that the arguments and Declaration presented obviate the Examiner's rejection under 35 U.S.C. §112, first paragraph. Accordingly, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. §112 first paragraph.

CONCLUSION

In view of the foregoing amendments and remarks, claims 22-43 should now be in condition for allowance. A notice to this effect is respectfully requested. If the Examiner believes, after this amendment, that the application is not in condition for allowance, the Examiner is requested to call the Applicant's attorney at the telephone number listed below.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, that is not covered by an enclosed check, please charge any deficiency to Deposit Account No. 23/2825.

Respectfully submitted,
Arthur M. Krieg et al., Applicant

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x10/02/03x

Exhibit 1

IFN-alpha secretion from human PBMC after incubation with ODN and IL-3
Human PBMC from two different donors were incubated for 24h with ODN 2006 and IL-3 at concentrations as indicated. Culture supernatant was harvested and IFN-alpha levels were measured by ELISA. Shown are the calculated mean values of both donors.

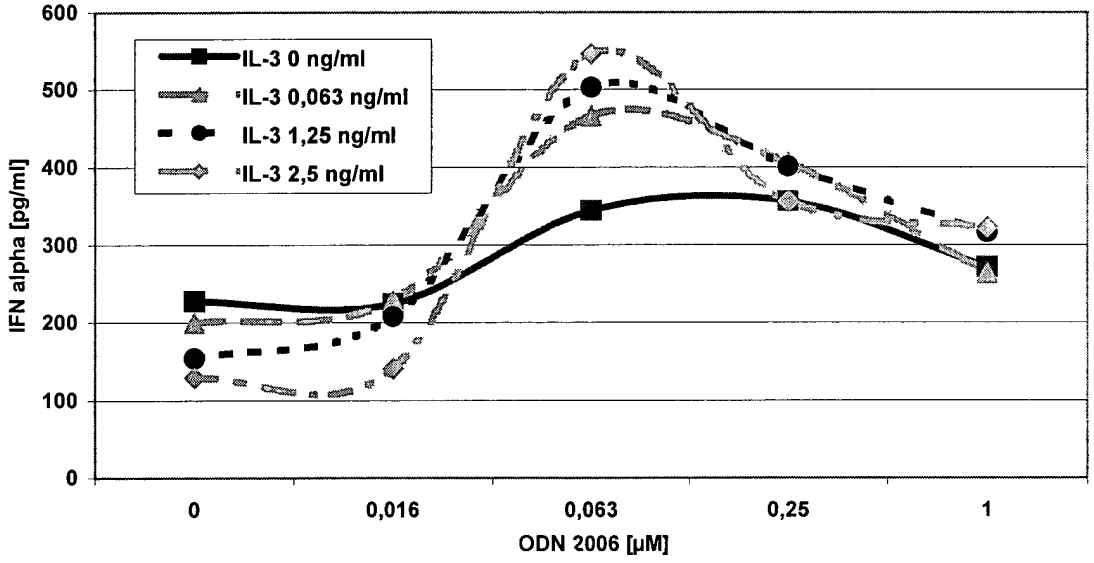


Exhibit 2

IFN-gamma secretion from human PBMC after incubation with ODN and IL-12
Human PBMC from three different donors were incubated for 24h with ODN and IL-12 at concentrations as indicated. Culture supernatant was harvested and IFN-gamma levels were measured by ELISA. Shown are the calculated mean values of all three donors.

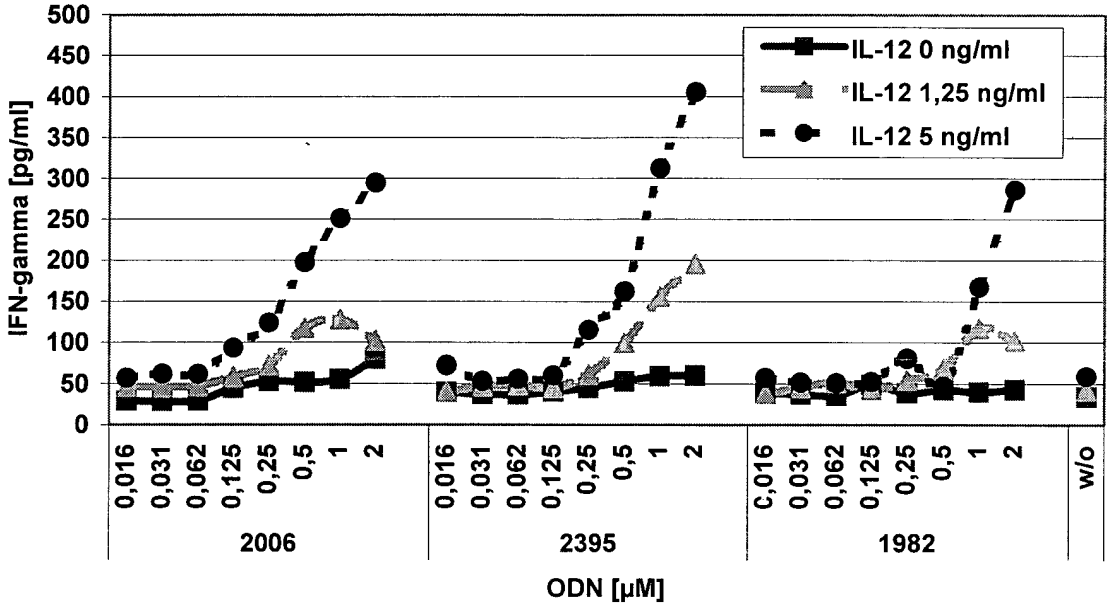
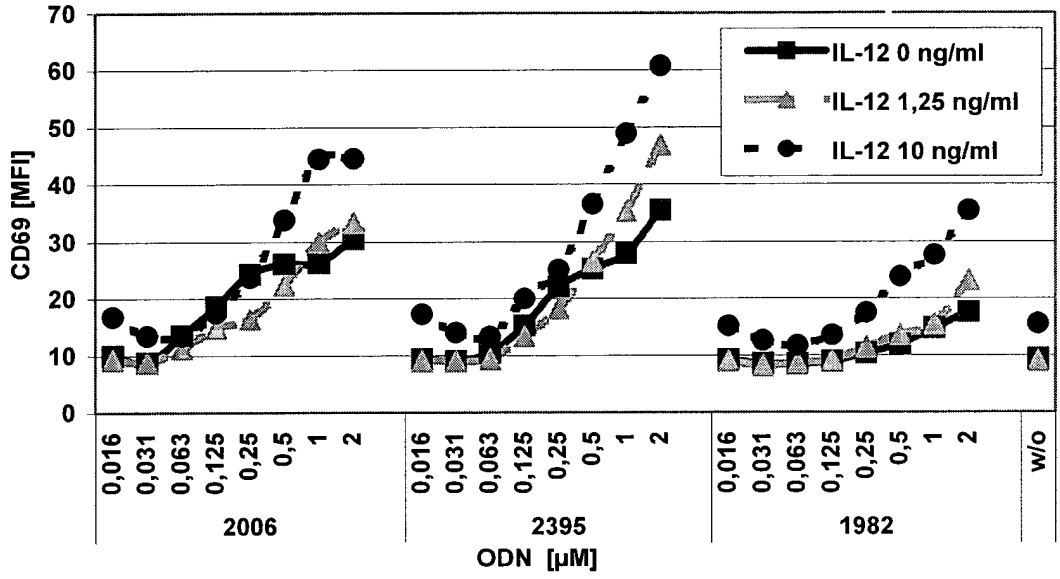


Exhibit 3

CD69 expression on human NK cells after incubation with ODN and IL-12
Human PBMC from three different donors were incubated with ODN and IL-12 at concentrations as indicated for 24h. Cells were harvested and stained with fluorochrome conjugated antibodies. Shown is the calculated mean of the measured mean fluorescence intensity values for CD69 of all three donors.



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CpG-containing oligodeoxynucleotides augment and switch the immune responses of cattle to bovine herpesvirus-1 glycoprotein D☆

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Abstract

The adjuvanticity of a synthetic oligodeoxynucleotide containing unmethylated CpG motifs (CpG ODN) was determined in cattle. Calves were immunized with a truncated secreted version of glycoprotein D (tgD) of bovine herpes virus-1 (BHV-1) formulated with alum, CpG ODN, or a combination of both. BHV-1 tgD formulated with CpG ODN or with alum and CpG ODN induced a stronger and more balanced immune response than tgD in alum. This level of immunity was of sufficient magnitude to minimize weight loss and significantly reduce the duration of virus shedding after intranasal viral challenge. Local tissue reactions generated by CpG ODN were very mild and transient, whereas reactions induced by alum or a combination of CpG ODN and alum were moderate in severity and duration. These data demonstrate that CpG ODN causes minimal injection site reactions and yet acts as an effective adjuvant in cattle.

Keywords: CpG-oligodeoxynucleotides; Adjuvant; Cattle

1. Introduction

Bovine herpes virus-1 (BHV-1) is an important pathogen in cattle, which causes a variety of clinical manifestations including rhinotracheitis, conjunctivitis, vulvovaginitis and abortions. Additionally, secondary opportunistic bacterial infections can lead to enhanced morbidity and mortality [1]. Previously, we have shown that the major glycoproteins of BHV-1, gB, gC and gD, induce protection in cattle when administered as a subunit vaccine formulated with an oil-based adjuvant [2]. However, as oil-based adjuvants also result in significant losses due to meat trimming, there is a need within the livestock industry to move towards adjuvants that induce minimal injection site reactions of short duration. Oil-based adjuvants also induce primarily Th2-type immune responses [3,4], which often are not protective and sometimes may be detrimental [5,6].

Recently, the potential of using bacterial DNA as an adjuvant has been demonstrated in mice and primates [7,8]. In bacterial DNA, CpG dinucleotides are unmethylated and present at an expected frequency (1/16 bases) whereas they are under-represented (1/50 to 1/60 bases) and selectively methylated in vertebrate genomes [9]. Because of these differences, it has been suggested that vertebrates recognize bacterial DNA as foreign due to their evolutionary adoption of a non-self pattern recognition mechanism [10]. Immunostimulatory DNA sequences have been further studied in the form of synthetic oligodeoxynucleotides (ODN). In vitro, ODN containing CpG motifs can activate murine macrophages as demonstrated by their secretion of IL-12, TNF-α, IFN-α and IFN-β [11,12]. CpG ODNs are also mitogenic for B cells [13], can stimulate dendritic cells [14], and indirectly induce NK cells to secrete IFN-γ and enhance their lytic activity [15,16].

In mice, CpG ODN is a potent adjuvant when administered with an antigen. Furthermore, when combined with other adjuvants such as alum or Freund's incomplete adjuvant (FIA), CpG ODN contributes to a synergistic immunostimulatory effect, which can enhance immune responses to a level comparable to that induced by Freund's complete adjuvant (FCA) [8,17,18]. In contrast to oil-based adjuvants, CpG ODN promotes the generation of Th1-biased immune

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responses [19,20] and elicits antigen-specific cellular and humoral immune responses that are far superior to those induced by alum [18], which is still the only adjuvant licensed for human use in many countries. In mice there is remarkably little injection site reaction or other toxicity associated with CpG ODN when employed as an adjuvant [8]. In addition, CpG DNA has strong immunomodulatory and immunostimulatory activity when applied as a therapy in murine models of allergy and cancer [21].

The potential of CpG ODN as an adjuvant has also been examined in large outbred animals. In Aotus monkeys, the antigenicity of a candidate malaria peptide vaccine was greatly enhanced by addition of CpG ODN to the formulation [22]. Orangutans are naturally infected with hepatitis B virus (HBV), but do not respond well to Energix-B® (SmithKline-Beecham, Rixensart, Belgium), a commercial HBV vaccine. The addition of CpG ODN to the Energix-B® formulation increased the level of seroprotection conferred by the vaccine from 8 to 100% after two immunizations [7].

Previously, we identified a CpG motif that elicits in vitro proliferative responses of bovine lymphocytes [23]. Since in vitro proliferation induced by an ODN is indicative of in vivo adjuvant potential, the most stimulatory ODN, which contains three copies of this motif, was chosen to adjuvant a truncated, secreted form of glycoprotein D (tgD) [24] in a BHV-1 challenge trial. Calves were immunized with tgD formulated with alum, CpG ODN or a combination of both. As reported for mice, CpG ODN alone or in combination with alum generated stronger and more balanced immune responses than alum, yet caused mild and transient tissue reaction. Furthermore, enhanced protection was observed, demonstrating that even in large animals, CpG DNA has potential as an adjuvant.

2. Materials and methods

2.1. Cells, viruses and glycoproteins

Bovine viral diarrhea virus-free Madin Darby bovine kidney (MDBK) cells were cultured in minimal essential medium (MEM; Gibco-BRL, Grand Island, NY), supplemented with 5% fetal bovine serum (FBS; Gibco-BRL). The 108 strain of BHV-1 was propagated in these cells. Virus recovered from nasal fluids was quantified by plaque titration on MDBK cells in microtiter plates with an antibody overlay as previously described [25]. A truncated secreted version of BHV-1 gD (tgD) was constructed by terminating the protein at amino acid 355, immediately upstream of the transmembrane anchor. Truncated gD was expressed in MDBK cells under regulation of the bovine heat shock 70A (hsp70) gene promoter [24] and purified as described previously [26]. Authentic gD used for in vitro stimulation of peripheral blood mononuclear cells (PBMC) was purified as described previously [2].

2.2. Experimental animals

Thirty 8–9-month-old healthy Hereford calves were purchased from a local ranch and housed in isolation at the research station of the Veterinary Infectious Disease Organization. These calves had never been vaccinated against BHV-1 and they were sero-negative to BHV-1. Calves were ranked according to weight and then randomly allocated to one of six treatment groups of five animals each. The average weight of the calves was 258 kg.

2.3. Immunizations and challenge

Six groups of calves were immunized twice intramuscularly in the neck with one of the following formulations: (1) Placebo (PBS); (2) BHV-1 tgD with 10 mg of ODN 2135 and alum; (3) BHV-1 tgD with 2 mg of ODN 2135 and alum; (4) BHV-1 tgD with 0.4 mg of ODN 2135 and alum; (5) BHV-1 tgD with 10 mg of ODN 2135; or (6) BHV-1 tgD in alum. All animals were re-immunized 4 weeks later. ODN 2135 (5′-TCGTCGTTTGTCGTTTTGTCGTT-3′) has a phosphorothioate backbone and out of a panel of 38 ODNs, this ODN had the strongest immunostimulatory ability for bovine lymphocytes in vitro [23]. The dose of BHV-1 tgD was 20 μg per animal. In formulations containing alum, antigen was absorbed onto 40 μg of the adjuvant. All alum and ODN formulations were prepared in a volume of 1 ml, with phosphate-buffered saline (PBS: 0.1 M NaH_2PO_4/Na_2HPO_4, 0.15 M NaCl, pH 7.8) as the diluent. Five weeks after the second immunization, the calves in groups 1, 2, 5 and 6 were each exposed for 4 min to an aerosol of 10^7 pfu per ml of BHV-1 strain 108, which was generated by a Devilbis Nebulizer, model 65 (DeVilbis, Barrie, ON).

2.4. Sampling

Sera were collected biweekly after each immunization and again 1 and 4 weeks post challenge. Peripheral blood was collected in citrate-dextran 1 week after BHV-1 challenge. Nasal tampons were used to obtain up to 5 ml of nasal fluid from all animals 3 days before challenge, as well as on days 2, 4, 6, 8, 10 and 13 post challenge.

2.5. Enzyme linked immunosorbent assays (ELISAs)

Polystyrene microtiter plates (Immulon 2, Dynatech Laboratories, Gaithersburg, MD) were coated with 0.5 μg of BHV-1 per well and incubated with serially diluted bovine sera, starting at 1:10 in four-fold dilutions. Alkaline phosphatase (AP)-conjugated goat anti-bovine IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a dilution of 1:10,000 was used to detect IgG. Antibody isotypes were determined in an indirect ELISA using tgD-coated plates, IgG1 and IgG2-specific monoclonal antibodies (provided by

Dr. K. Nielson, Canadian Food Inspection Agency, Ottawa, ON) and affinity-purified, AP-conjugated rabbit anti-mouse IgG (Kirkegaard and Perry Laboratories) at a dilution of 1:10,000. The reactions were visualized with *p*-nitrophenyl phosphate (PNPP) (Sigma, Oakville, ON).

2.6. Virus neutralization assays

The neutralization titers in the sera were determined as previously described. The titers were expressed as the highest dilution of antibody that caused a 50% reduction of plaques relative to the virus control [2].

2.7. Proliferation assays

Bovine blood was collected into citrate-dextran and PBMCs were isolated on Ficoll-Paque PLUS (Pharmacia, Mississauga, ON). PBMCs were dispensed at 3.5×10^6 cells/ml of culture medium consisting of MEM (Gibco-BRL), 10 % FBS (Sigma), 2 mM L-glutamine (Gibco-BRL), 500 mg/ml gentamycin, 5×10^{-5} M 2-mercaptoethanol and 1 mg/ml dexamethasone. Subsequently, 100 μl volumes were dispensed into the wells of microtiter plates. Purified gD [2] at 1 μg/ml was added in a 100 μl volume to triplicate wells. Another three wells contained cells without antigen. After 3 days in culture at 37 °C in a humidified CO_2 incubator, the cells were pulsed with [methyl-^{3}H]-thymidine (Amersham, Oakville, ON) at a concentration of 0.4 μCi/well. The cells were harvested 18 h later and thymidine uptake was measured by scintillation counting. Proliferative responses were calculated as the means of triplicate wells and expressed as a stimulation index (SI) (counts/min in the presence of antigen divided by counts/min in the absence of antigen). The stimulation indices per group were calculated as the arithmetic average SI.

2.8. IFNγ capture assays

Antigen-specific IFNγ secretion was detected using an immunosorbent assay. PBMCs were suspended in MEM (Gibco-BRL), 10 % FBS (Sigma), 2 mM L-glutamine (Gibco-BRL), 500 mg/ml gentamycin, 5×10^{-5} M 2-mercaptoethanol and 1 mg/ml dexamethasone, and plated in triplicate at a density of 1×10^6 cells/well in the presence or absence of 0.4 μg/ml gD in sterile polystyrene microtiter plates (Immulon 2, Dynatech Laboratories) coated with a monoclonal antibody against bovine IFNγ (clone 2.2.1) [27]. Following a 48 h incubation at 37 °C in a humidified CO_2 incubator, cells were removed from the plates by washing in cold double distilled water. Secreted, bound IFNγ was detected by incubation with rabbit anti-bovine IFNγ serum (lot 92–131) [27], followed by incubation with AP-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories) and subsequent development with PNPP (Sigma). The amounts of antigen-specific IFNγ were calculated using doubling dilutions of IFNγ, which were present on all plates with a range of 4 ng/ml to 20 pg/ml. The amount of spontaneously secreted IFNγ was subtracted from the amount of antigen-specific IFNγ to arrive at the amount of gD-specific cytokine secretion.

2.9. Tissue samples

ODN 2135 (1 mg), alum, alum + ODN 2135 (1 mg), LPS (10 μg) or saline was delivered intradermally by Biojector (Bioject Inc., Portland, OR) in a 100 μl volume. All samples were delivered in three different animals for each time point in order to obtain triplicate values. At the different time points after delivery of the adjuvant, tissue biopsies, 8 mm in diameter, were taken from the injection sites. They were fixed in 10% formalin buffer for 12 h, embedded in paraffin, sectioned at 5 μm thickness and stained with haematoxylin and eosin (HE). Inflammation was assessed according to a scoring system based on tissue reactions to the various adjuvants, with values ranging from 0 to 4.

2.10. Statistical analyses

All data were analyzed with the aid of a statistical software program (Systat 7.0, SPSS Inc., Chicago, IL). ELISA and VN data were transformed prior to performing the analysis by log transformation, because they were not normally distributed. Differences between the groups were examined by performing the one-way ANOVA and Dunnett's test for SI's and ELISPOT counts and the two-way ANOVA and Tukey HSD multiple comparison for ELISA titers, VN titers, weights and virus shedding.

3. Results

3.1. Humoral immune responses induced by tgD

In order to assess the adjuvant activity of a CpG containing ODN in cattle, three groups of calves were vaccinated with BHV-1 tgD formulated with a combination of alum and different doses of ODN 2135. Two additional groups received either tgD in alum or tgD with the highest dose of ODN 2135. After one immunization there was no significant increase in total IgG, but after the second immunization all vaccinated groups, except the group immunized with tgD in alum, had significantly higher titers than the placebo group ($P < 0.05$) (Fig. 1A). Although the titers decreased somewhat over time, the groups that received tgD and 10 mg ODN or tgD, alum and10 mg ODN still had higher titers ($P < 0.05$) than the placebo group 3 weeks later. In contrast, the titers of the other vaccinated groups were not different from those of the placebo group at that time. In order to assess the biological activity of the BHV-1 specific antibodies, VN assays were carried out. All vaccinated groups developed virus neutralizing antibodies, and the differences in VN titers between the groups were similar

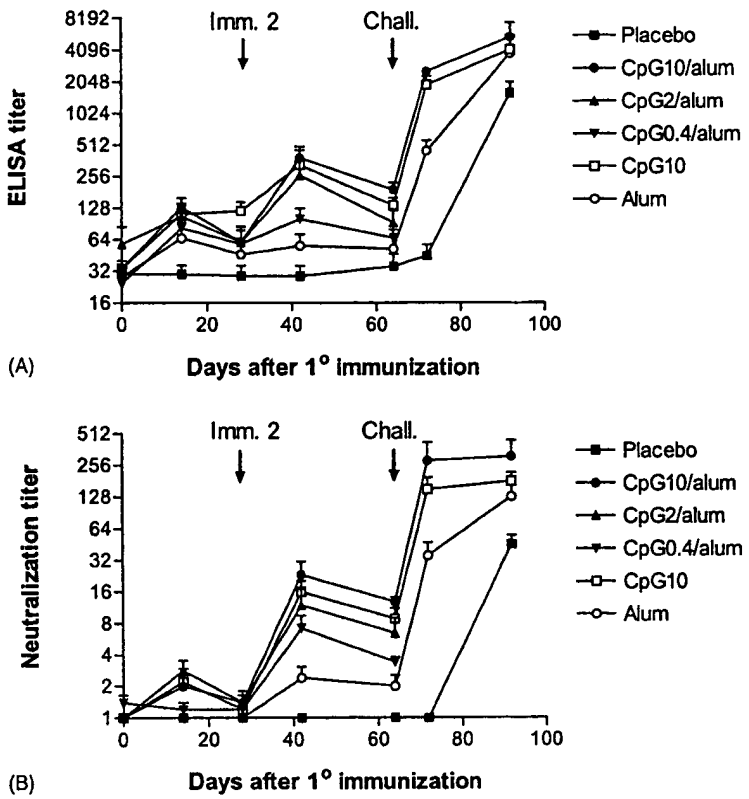

Fig. 1. Serum antibody responses. Calves were immunized twice IM with (1) placebo; (2) tgD, alum and 10 mg of ODN 2135 (CpG10/alum); (3) tgD, alum and 2 mg of ODN 2135 (CpG2/alum); (4) tgD, alum and 0.4 mg of ODN 2135 (CpG0.4/alum); (5) tgD with 10 mg of ODN 2135 (CpG10); or (6) tgD in alum. Five weeks after the secondary immunization the calves in groups 1, 2, 5 and 6 were challenged intranasally with BHV-1. (A) BHV-1-specific ELISA titers expressed as the reciprocal of the highest dilution resulting in an OD reading of greater than mean plus two standard deviations above the control value; (B) serum virus neutralization titers expressed as a 50 % endpoint using 100 PFU of BHV-1. Error bars show the standard deviation of the means.

to those in total IgG titers (Fig. 1B). Previous studies have shown that tgD formulated with an oil-based adjuvant tends to induce primarily IgG1 and very little if any IgG2 [26]. Since according to published observations in the mouse model CpG ODN generally induce Th1-type responses, the isotype ratios were determined. As illustrated in Fig. 2A, the use of alum also leads to a strong excess of IgG1 over IgG2, whereas increasing concentrations of CpG ODN tended to increase the IgG2:IgG1 ratio in a dose-dependent manner.

As the calves immunized with tgD and 10 mg of ODN 2135 or tgD, alum and10 mg ODN 2135 developed the strongest neutralizing antibody response, these groups of animals, as well as the placebo and alum groups, were challenged with BHV-1. One week after challenge, all vaccinated groups had significantly higher BHV-1 specific total IgG and VN titers than the placebo group ($P < 0.001$), and the groups vaccinated with tgD, alum and10 mg ODN had significantly higher titers than the group that received tgD in alum alone ($P < 0.05$) (Fig. 1A, B). After challenge, an IgG1:IgG2 ratio of approximately 1:1 was observed in the groups that were immunized with tgD and ODN 2135 with or without alum, whereas in the groups that were not vaccinated or immunized with tgD and alum the IgG1:IgG2 ratios were 2 and 3, respectively (Fig. 2B).

3.2. Cellular immune responses induced by tgD

In order to assess the presence of cellular immune responses, PBMCs were isolated from peripheral blood after challenge, and stimulated in vitro with BHV-1 gD. Antigen-specific responses were measured by cellular proliferation. As illustrated in Fig. 3A, the animals vaccinated with tgD and ODN 2135 or a combination of alum and ODN 2135 tended to have higher antigen-specific proliferative responses than the calves in the placebo group, whereas there was no difference between the alum group and the placebo group. Although three animals in the group that received tgD with ODN 2135 also had a higher stimulation index, the difference between this group and the placebo group was not significant (Fig. 3B). To confirm that T cells were activated, the production of IFNγ was assessed. Similar to the lymphocyte proliferative response, the group vaccinated with tgD, alum and ODN 2135 showed a significantly higher amount of IFNγ secretion by the PBMCs than the placebo group

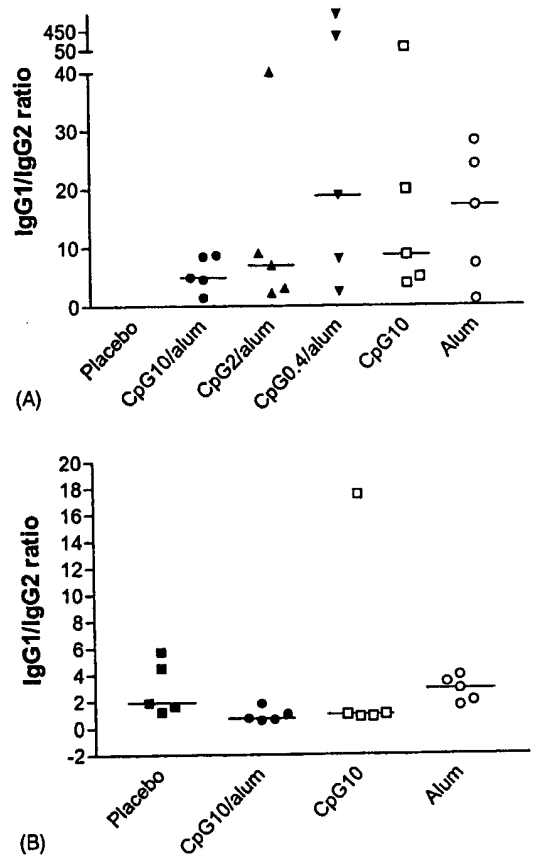

Fig. 2. IgG subtype ratio's. Calves were immunized twice IM with (1) placebo; (2) tgD, alum and 10 mg of ODN 2135 (CpG10/alum); (3) tgD, alum and 2 mg of ODN 2135 (CpG2/alum); (4) tgD, alum and 0.4 mg of ODN 2135 (CpG0.4/alum); (5) tgD with 10 mg of ODN 2135 (CpG10); or (6) tgD in alum. Five weeks after the secondary immunization the calves in groups 1, 2, 5 and 6 were challenged intranasally with BHV-1. (A) IgG1:IgG2 ratio's prior to BHV-1 challenge; (B) IgG1:IgG2 ratio's after BHV-1 challenge. Bars represent the median value.

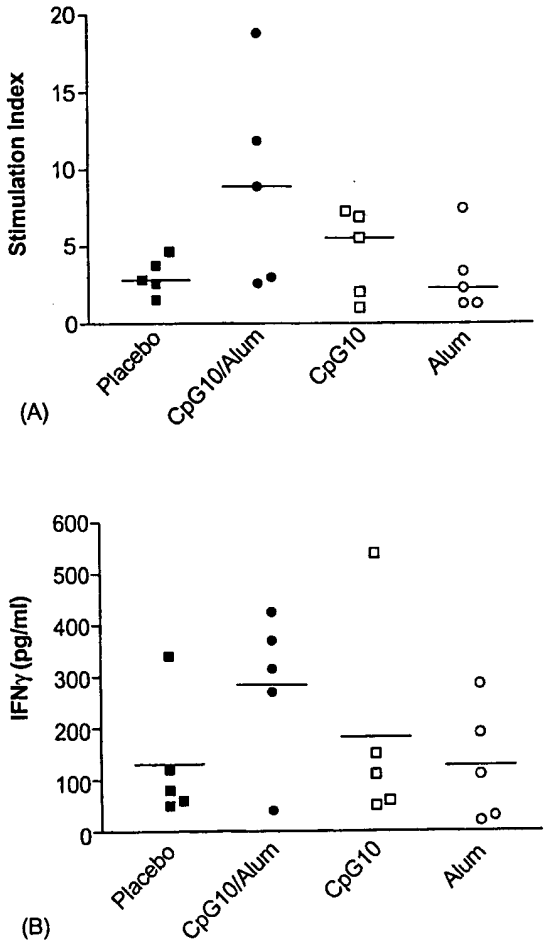

Fig. 3. Cellular immune responses. Calves were either left untreated as negative controls (placebo) or immunized twice IM with tgD, alum and 10 mg of ODN 2135 (CpG10/alum); tgD with 10 mg of ODN 2135 (CpG10); or tgD in alum. Five weeks after the secondary immunization the calves were challenged intranasally with BHV-1. (A) Proliferative responses were measured 1 week after challenge following in vitro stimulation with 1 μg/ml of gD. The results represent the average of triplicate wells and are shown for each animal, and as the mean stimulation index (SI) for each group of animals; (B) the amount of IFNγ secretion was measured 1 week after challenge following in vitro stimulation of PBMCs with 1 μg/ml of gD. The results represent the average of triplicate wells and are shown for each animal, and as the average amount of IFNγ secretion for each group of animals.

($P < 0.05$). The wide distribution of the values for stimulation index and amount of IFNγ secretion for these animals is due to their out-bred nature, which causes each animal to respond with a different kinetics of T cell proliferation.

3.3. Clinical observations after challenge

One of the more objective indicators for the well-being of an animal and the level of protection achieved is the amount of weight loss measured post challenge. With exception of the first day when the animals appeared to gained some weight due to shrinkage on the challenge day and subsequent food and water intake, all animals lost weight (Fig. 4A). However, the animals in the groups vaccinated with tgD, alum and 10 mg of ODN 2135 or tgD and 10 mg of ODN 2135 regained their weight more quickly than the placebo or alum groups. As a further objective measurement of protection, the amount of virus recovered from the nasal secretions was measured over a 2-week period after challenge. Nasal virus shedding was observed from day 2 onwards in all groups. The maximum level of virus shedding in the placebo group occurred on day 6 post challenge, and considerable shedding was still observed on day 10. In contrast, the nasal shedding in the vaccinated groups rapidly decreased from day 6 onwards and overall was significantly lower than in the control group ($P < 0.01$) (Fig. 4B).

3.4. Local effects of CpG ODN and alum

One of the concerns about some adjuvants, specifically those that are oil-based, is the occurrence of local tissue

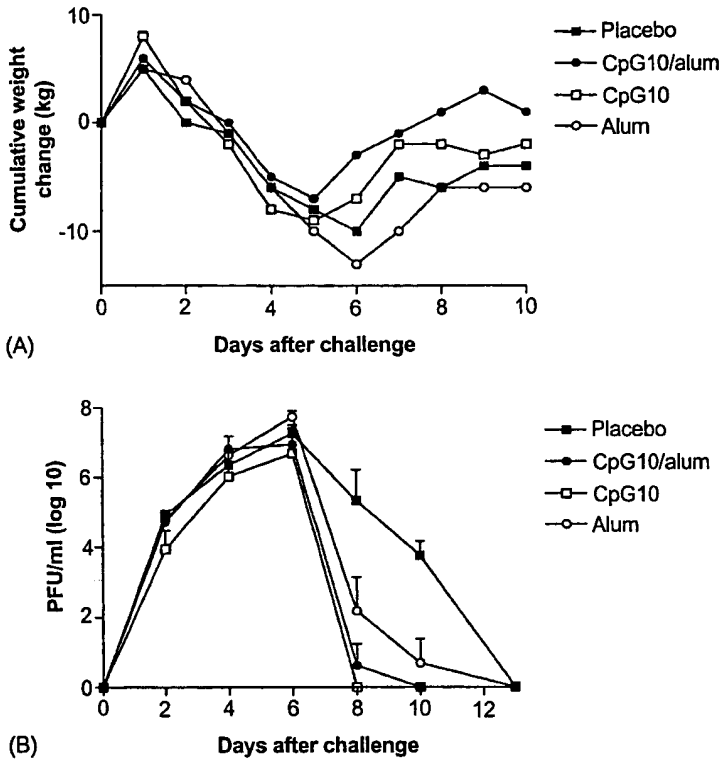

Fig. 4. Clinical course after challenge with BHV-1. Calves were either left untreated as negative controls (placebo) or immunized twice IM with tgD, alum and 10 mg of ODN 2135 (CpG10/alum); tgD with 10 mg of ODN 2135 (CpG10), or tgD in alum. Five weeks after the secondary immunization the calves were challenged intranasally with BHV-1. (A) Cumulative weight change; (B) nasal shedding of BHV-1. Error bars show the standard deviation of the means.

reactions. Unfortunately, there generally is a strong correlation between the efficacy of an adjuvant and the degree of tissue reaction. In order to assess local effects generated by the adjuvants used in the current trial, ODN 2135, alum or alum + ODN 2135 were delivered into the skin. LPS and PBS were injected similarly as positive and negative controls. The scoring criteria are illustrated in Fig. 5A–E and 5G. The physical force of the Biojector mediated delivery caused some focal tissue damage (Fig. 5F), characterized by focal necrosis with moderate PMN infiltration. This lesion was disregarded for the scoring. As predicted, saline had no effect at all, whereas LPS caused severe superficial and deep perivascular dermatitis with diffuse PMN infiltration in the superficial and deep dermis (Fig. 6). This effect was observed as early as 6 h after injection, but it essentially disappeared by day 3. In contrast, CpG ODN caused transient and mild focal or multifocal perivascular dermatitis with local PMN infiltration in the superficial and deep dermis. Alum also caused mild dermatitis between 6 and 48 h after delivery, but between days 6 and 14, moderate to severe superficial and deep perivascular dermatitis and PMN infiltration in the superficial and deep dermis was observed. Similarly, from 6 h onwards the combination of alum and CpG ODN resulted in moderate to severe dermatitis. Importantly, by day 14 the inflammation caused by alum or alum and CpG ODN started to diminish.

4. Discussion

This is the first report demonstrating the potential of CpG DNA to enhance immune responses against an economically important pathogen in a veterinary species, as to date only rodent or primate models have been used to demonstrate the efficacy of this class of immunostimulant. These data demonstrate that ODN 2135 has an adjuvant effect when formulated at 10 mg per dose with BHV-1 tgD and that the addition of ODN 2135 to a tgD-alum formulation enhances the antibody titers in a dose-dependent manner in cattle. Furthermore, immunization with tgD and a combination of alum and ODN 2135 resulted in higher IgG2 to IgG1 subtype ratios and stronger cellular immunity than vaccination with tgD in alum alone. Finally, vaccination with tgD in any of the formulations tested resulted in significantly lower amounts of virus shedding post BHV-1 challenge.

The efficacy of a vaccine relies in large part upon its formulation. In contrast to the strong synergistic effect between alum and CpG ODN reported for HBsAg in mice [8,18], the addition of alum to a CpG ODN-tgD formulation did not significantly enhance the antibody responses induced with CpG ODN alone although the titers tended to be higher in the CpG/alum group. In the mouse study, there was a strong synergy of alum with CpG ODN, which was thought to be due to a depot effect, increasing the time that both the

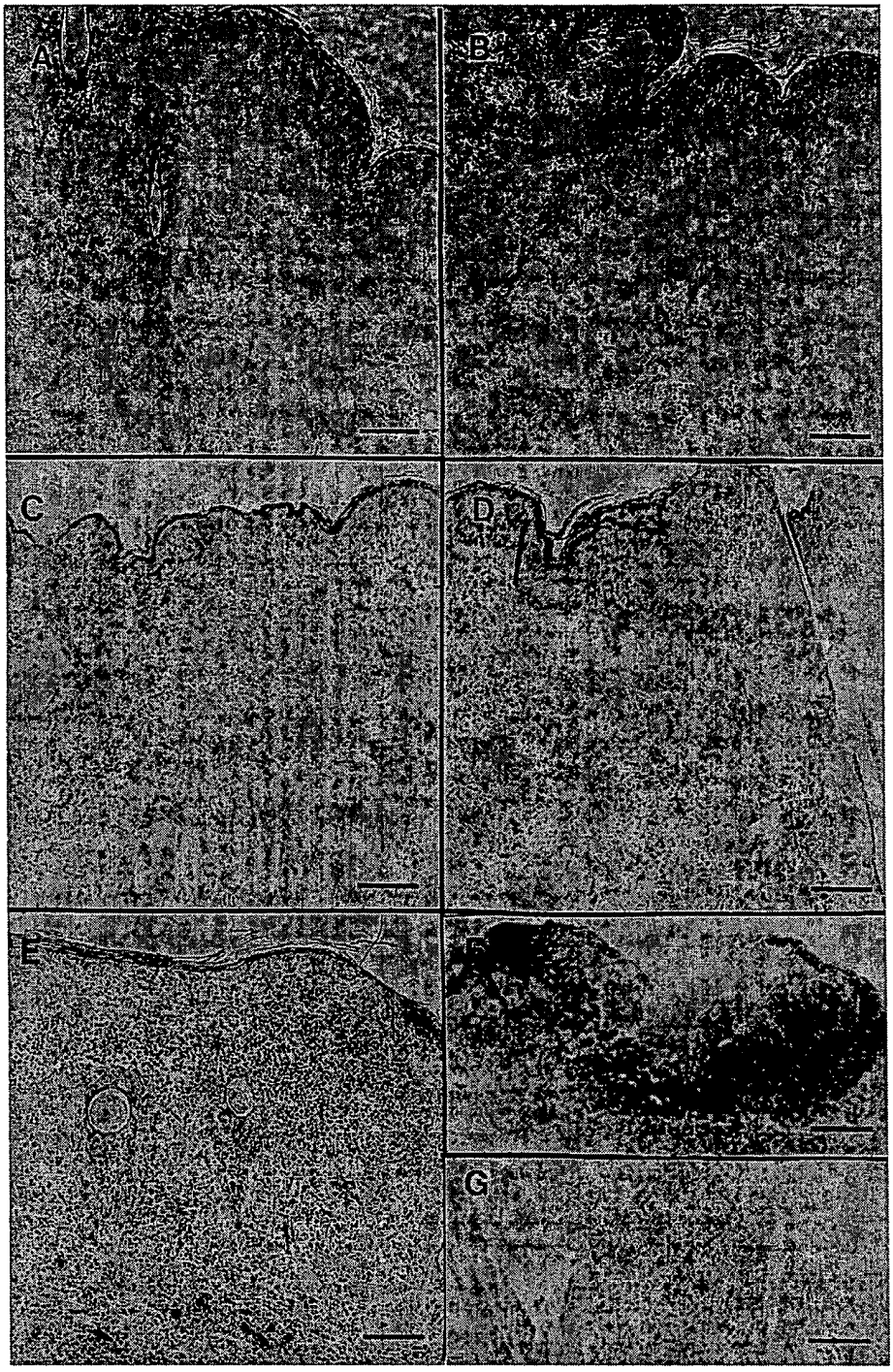

Fig. 5. Scoring index. Inflammation following adjuvant delivery was assessed according to a scoring system with values ranging from 0 to 4. (A) 0: normal; (B) 1: mild (mild, focal or multifocal superficial perivascular dermatitis with PMN infiltration in the superficial dermis); (C) 2: moderate (moderate, multifocal to locally extensive superficial perivascular dermatitis with PMN infiltration in the superficial dermis); (D) 3: moderate to severe (moderate to severe superficial and deep perivascular dermatitis with locally extensive PMN infiltration in the superficial and deep dermis); (E) 4: severe; superficial section (severe superficial and deep perivascular dermatitis with diffuse PMN infiltration in the superficial and deep dermis); (F) tissue damage due to injection, which was disregarded for the scoring system. Microscopic lesions with focal necrosis and moderate PMN filtration; (G) 4: severe; deep section (severe superficial and deep perivascular dermatitis with diffuse PMN infiltration in the superficial and deep dermis). Photographs were taken with an Olympus AH2-RFL microscope using standard light. Bar = 1 μm.

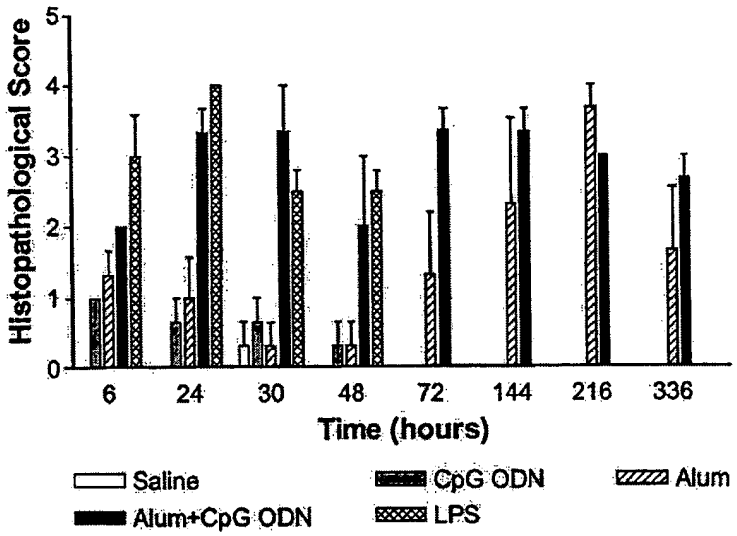

Fig. 6. Histopathological scores. Calves were injected with saline, ODN 2135 (CpG ODN), alum, alum + ODN 2135 (CpG ODN), or LPS. Punch biopsies were taken at different time points after delivery, sectioned and treated with HE staining. The level of tissue damage was assessed by giving histopathological scores between 0 and 4. All observations represent the average ± S.E.M. of three biopsies.

antigen and the CpG ODN are available. However, we recently showed that alum may not be as good an adjuvant for tgD due to minimal binding of the protein to the adjuvant, so this may have had an impact upon the magnitude of responses. Although there was no significant effect of the addition of alum on the magnitude of the antibody response, the combination of alum and CpG ODN appeared to influence the IgG2:IgG1 ratio's and the cellular immune responses, which were higher in the animals immunized with the combination of adjuvants. These animals also regained their weights more quickly, indicating a faster recovery. The enhanced cellular immunity as well as the increased IgG2:IgG1 ratio agrees with observations made for mice, where the addition of CpG ODN resulted in a shift towards a more Th1-type response. Although these and previous studies suggest that this may not be essential for protection from BHV-1 challenge [28], the induction of strong cellular immunity is critical for protection against several other diseases such as those caused by intracellular bacteria and some viruses like hepatitis B virus, human immunodeficiency virus and human and bovine respiratory syncytial virus [5,6]. A combination of alum and CpG ODN could be very effective for the induction of protective immunity from these types of pathogens.

The observation that the presence of alum in the tgD-CpG ODN formulation appeared to enhance the immune responses to a certain extent indicates the need for a co-adjuvant to generate a depot effect. This is specifically important in large animals such as cattle where antigen and CpG ODN may diffuse and traffic to different draining lymph nodes if not co-administered with a an adjuvant that traps both antigen and CpG ODN at the site of administration. However, alum probably is not the optimal adjuvant for CpG ODN-tgD formulations, so in order to optimize the use of CpG ODN as an adjuvant in cattle, additional co-adjuvants need to be tested. Other CpG ODN adjuvant combinations that work synergistically have been reported including FIA in mice [8,18] and Montanide 720 in monkeys [22], so it should be feasible to select an adjuvant that is effective with BHV-1 tgD, and combine this with ODN 2135 to induce enhanced immune responses.

In addition to evaluating the immune responses and protection induced by adjuvanting with CpG ODN, the effect of this type of adjuvant on local tissues was assessed histologically. In contrast to LPS, a known inducer of inflammation, CpG ODN caused a mild and transient reaction. Importantly, although the adjuvanticity of alum was enhanced by the addition of CpG ODN, an increase in tissue damage was only observed during the first 2 days after delivery, when CpG ODN and alum each caused some dermatitis, which resulted in an additive effect when administered together. As the damage caused by CpG ODN was transient, from day 3 onwards there was no difference between the tissue reaction to CpG ODN alone and the reaction to CpG ODN and alum combined. In the mouse model, gross pathological examination of tissues injected with different adjuvants and combinations thereof were performed on day 7 after delivery. This experiment demonstrated that FCA and Titermax, both oil-based, caused extensive tissue damage, whereas FIA, MPL and CpG ODN caused moderate to mild damage and alum hardly caused any reaction [8]. Some combinations with CpG ODN, for example with FIA, caused more damage than the individual adjuvants. Most interestingly, whereas the degree of damage generally was proportional to the adjuvant activity when the individual adjuvants were used, the combination

of alum and CpG ODN induced strong HBsAg-specific immune responses with minimal damage, which agrees with our observations between days 6 and 14 in cattle.

In conclusion, a previously identified CpG ODN with mitogenic properties for bovine PBMCs in vitro was shown to have adjuvant capabilities in a BHV-1 vaccination and challenge experiment. Continuing research aimed at optimizing doses, formulations and delivery of CpG ODN with a view to enhancing antigen-specific immune responses in cattle is in progress. Furthermore, a dose has been identified which will allow further examination of CpG immunostimulation or adjuvanticity in cattle and other large animals.

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References

[1] Yates WDG. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can J Comp Med 1982;46:225–63.
[2] van Drunen Littel-van den Hurk S, Gifford GA, Babiuk LA. Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. Vaccine 1990;8(4):358–68.
[3] Singh M, Carlson JR, Briones M, et al. A comparison of biodegradable microparticles and MF59 as systemic adjuvants for recombinant gD from HSV-2. Vaccine 1998;16:1822–7.
[4] Valensi JP, Carlson JR, Van Nest GA. Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other adjuvants. J Immunol 1994;153:4029.
[5] Kimman TG, Sol J, Westenbrink F, Straver PJ. A severe outbreak of respiratory tract disease associated with bovine respiratory syncytial virus probably enhanced by vaccination with modified live virus. Vet Quart 1989;11:250–3.
[6] Kim HW, Canchola JG, Brandt CD, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 1969;89:422–34.
[7] Davis HL, Suparto II, Weeratna RR, et al. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine 2000;18(18):1920–4.
[8] Weeratna RD, McCluskie MJ, Xu Y, Davis HL. CpG DNA induces stronger immune responses with less toxicity than other adjuvants. Vaccine 2000;18(17):1755–62.
[9] Cardon LR, Burge C, Clayton DA, Karlin S. Pervasive CpG suppression in animal mitochondrial genomes. Proc Natl Acad Sci USA 1994;91(9):3799–803.
[10] Krieg AM, Yi A-K, Schorr J, Davis HL. The role of CpG dinucleotides in DNA vaccines. Trends Microbiol 1998;6(1):23–7.
[11] Chace JH, Hooker NA, Mildenstein KL, Krieg AM, Cowdery JS. Bacterial DNA-induced NK cell IFN-gamma production is dependent on macrophage secretion of IL-12. Clin Immunol Immunopathol 1997;84(2):185–93.
[12] Sun S, Zhang X, Tough DF, Sprent J. Type I interferon-mediated stimulation of T cells by CpG DNA. J Exp Med 1998;188(12):2335–42.
[13] Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B cell activation. Nature 1995;374(6522):546–9.
[14] Sparwasser T, Koch ES, Vabulas RM, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol 1998;28(6):2045–54.
[15] Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol 1996;157(5):1840–5.
[16] Cowdery JS, Chace JH, Yi A-K, Krieg AM. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. J Immunol 1996;156(12):4570–5.
[17] Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine 1998;16(11/12):1216–24.
[18] Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 1998;160(2):870–6.
[19] Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J Exp Med 1997;186(10):1623–31.
[20] Lipford GB, Bauer M, Blank C, Reiter R, Wagner H, Heeg K. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur J Immunol 1997;27(9):2340–4.
[21] Klinman DM. Therapeutic applications of CpG-containing oligodeoxynucleotides. Antisense Nucl Acid Drug Dev 1998;8(2):181–4.
[22] Jones TR, Obaldia N, Gramzinski RA, et al. Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in Aotus monkeys. Vaccine 1999;17(23/24):3065–71.
[23] Pontarollo RA, Rankin R, Babiuk LA, et al. Monocytes are required for optimum in vitro stimulation of bovine peripheral blood mononuclear cells by non-methylated CpG motifs. Vet Immunol Immunopathol 2002;84:43–59.
[24] Kowalski JK, Gilbert SA, van Drunen Littel-van den Hurk S, van den Hurk JV, Babiuk LA, Zamb TJ. Heat-shock promoter-driven synthesis of secreted bovine herpesvirus glycoproteins in transfected cells. Vaccine 1993;11:1100–7.
[25] van Drunen Littel-van den Hurk S, Braun RP, Lewis PJ, et al. Intradermal immunization with a bovine herpesvirus-1 DNA vaccine induces protective immunity in cattle. J Gen Virol 1998;79:831–9.
[26] van Drunen Littel-van den Hurk S, Parker MD, Massie B, et al. Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. Vaccine 1993;11(1):25–35.
[27] Raggo C, Habermehl M, Babiuk LA, Griebel P. The in vivo effects of recombinant bovine herpesvirus-1 expressing bovine interferon-gamma. J Gen Virol 2000;81:2665–73.
[28] Loehr BI, Pontarollo R, Rankin R, et al. Priming by DNA immunization augments T cell responses induced by modified live bovine herpesvirus vaccine. J Gen Virol 2001;82:3035–43.

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Mary Ann Liebert, Inc.

CpG Motif Identification for Veterinary and Laboratory Species Demonstrates That Sequence Recognition Is Highly Conserved

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ABSTRACT

Oligodinucleotides containing CpG motifs stimulate vertebrate immune cells *in vitro*, have proven efficacy in murine disease models and are currently being tested in human clinical trials as therapies for cancer, allergy, and infectious disease. As there are no known immunostimulatory motifs for veterinary species, the potential of CpG DNA as a veterinary pharmaceutical has not been investigated. Here, optimal CpG motifs for seven veterinary and three laboratory species are described. The preferential recognition of a GTCGTT motif was strongly conserved across two vertebrate phyla, although a GACGTT motif was optimal for inbred strains of mice and rabbits. In a subsequent adjuvanticity trial, the *in vitro* screening methodology was validated in sheep, representing the first demonstration of CpG DNA efficacy in a veterinary species. These results should provide candidate immunostimulant and therapeutic drugs for veterinary use and enable the testing of CpG DNA in large animal models of human disease.

INTRODUCTION

CYTOSINE-PHOSPHATE-GUANOSINE (CpG) DINUCLEOTIDES are unmethylated and present at the expected frequency in bacterial DNA (1/16 bases), whereas they are underrepresented (1/50 to 1/60 bases) and selectively methylated in vertebrate genomes (Cardon et al., 1994). It has been suggested that because of these differences, a nonself pattern recognition mechanism has evolved in vertebrate immune systems enabling them to counter invading pathogens (Krieg et al., 1998).

The biologic activity of immunostimulatory DNA sequences can be further defined in the form of synthetic oligodeoxynucleotides (ODN). *In vitro*, an ODN containing a stimulatory motif can activate murine macrophages as demonstrated by their secretion of interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), and interferon-α/β (IFN-α/β) (Chace et al., 1997; Sun et al., 1998). CpG ODN are also mitogenic for B cells (Krieg et al., 1995), inhibit spontaneous B cell apoptosis (Yi et al., 1998), stimulate dendritic cells (DC) (Sparwasser et al., 1998), and indirectly induce natural killer (NK) cells to secrete IFN-γ and enhance their lytic activity (Ballas et al., 1996; Cowdery et al., 1996). *In vivo*, CpG DNA is a potent Th1 adjuvant (Lipford et al., 1997; Chu et al., 1997; Moldovenu et al., 1998; Davis et al., 1998; Weeratna et al., 2000), promoting antigen-specific cellular and humoral immune responses that are far superior to those induced by alum, which in many countries is the only adjuvant licensed for human use.

The potential of CpG ODN as an adjuvant has also been examined in large outbred animals. In Aotus monkeys, the antigenicity of a candidate malaria peptide vaccine was greatly enhanced by the addition of CpG ODN to the formulation (Jones et al., 1999). Orangutans, which are naturally infected with hepatitis B virus (HBV), do not respond well to Energix-B® (SmithKline Beecham Biologicals, Rixensart, Belgium), a

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commercial HBV vaccine. The addition of CpG ODN to the Energix-B formulation increased the level of seroprotection conferred by the vaccine in orangutans from 8% to 100% after two immunizations (Davis et al., 2000).

Interestingly, CpG DNA also has strong immunomodulatory and immunostimulatory activity when applied as a therapy in murine models of allergy and cancer. In allergen-sensitized mice, CpG DNA could prevent allergen-induced airway inflammation (Kline et al., 1998). Furthermore, in a model of murine allergic conjunctivitis, the systemic or mucosal administration of CpG DNA could inhibit allergic responses (Magone et al., 2000). Other studies have demonstrated that CpG DNA administration may lead to tumor rejection and can enhance the efficacy of monoclonal antibody (mAb) therapy against lymphoma, demonstrating the potential of CpG DNA as a valuable new drug with a broad array of potential uses (Wooldridge et al., 1997; Carpentier et al., 1999). Based on these and other promising data, a number of human clinical trials are in progress using CpG ODN as adjuvant or therapy for cancer and allergic conditions (Klinman, 1998; Krieg, 2000).

The optimal murine CpG motif (GACGTT) differs from that which best stimulates human and nonhuman primate immune cells (GTCGTT) and has little, if any, stimulatory activity on immune cells from humans, chimpanzees, Aotus monkeys, or cynomolgus monkeys (Jones et al., 1999; Davis et al., 2000; Hartmann et al., 2000). Based on these observations, it has been proposed that there is a degree of species specificity in the recognition of CpG motifs. Therefore, to be able to ascertain the *in vivo* potential for this technology in a target species, it is first essential to select CpG ODN that can optimally stimulate immune cells from the species of interest. As it is impractical to screen ODN *in vivo* in large animals, a selection must be based on *in vitro* activity using an appropriate assay.

In the current study, a proliferation assay, which has been shown to be a reliable predictive indicator of the *in vivo* adjuvant potential of an ODN (Hartmann et al., 2000), was used to screen a panel of ODN for *in vitro* biologic activity in 10 species. Although in inbred laboratory animals a GACGTT motif was optimal, the pattern of reactivity to CpG ODN was similar in all outbred species, with the major stimulatory motif being GTCGTT. The CpG ODN with the strongest *in vitro* stimulatory effect was successfully tested in an adjuvanticity trial in sheep. This represents the first report on immunostimulatory CpG motifs for a number of important livestock and companion animals. Our results suggest that CpG motif recognition is an ancient evolutionary adaptation that is highly conserved in a broad variety of species.

MATERIALS AND METHODS

ODN panel

A panel of 30 ODN was used in this study (Table 1). ODN with phosphorothioate (S-ODN) and phosphodiester (O-ODN) backbones were present in the panel of ODN. Recognizing that sequence elements of an ODN can influence its activity in a proliferation assay, the panel was broad in terms of motifs and other sequence elements. ODN lacking in CpG motifs were also tested. The panel of ODN tested *in vitro* was provided by Coley Pharmaceutical (Wellesley, MA), and ODN 2135 used *in vivo* was manufactured by Boston Biosystems (Bedford, MA). No endotoxin was detected in any of the ODN.

TABLE 1. SYNTHETIC ODN USED FOR SCREENING

ODN[a]	*Backbone*[b]	*Sequence*[c]
1585	SOS	GGGGTCAACGTTGAGGGG
2001	O	GGCGGCGGCGGCGGCGGCGG
2037	O	TCCATGCCGTTCCTGCCGTT
2038	O	TCCATGACGGTCCTGACGGT
2039	O	TCCATGCCGGTCCTGCCGGT
2040	O	TCCATGCGCGTCCTGCGCGT
2079	O	TCGACGTTCCCCCCCCCCCC
2080	O	TCGTCGTTCCCCCCCCCCCC
2082	O	TCGCCGTTCCCCCCCCCCCC
1760	S	ATAATCGACGTTCAAGCAAG
1826	S	TCCATGACGTTCCTGACGTT
1835	S	TCTCCCAGCGAGCGCCAT
1841	S	TCCATAGCGTTCCTAGCGGTT
1842	S	TCGTCGCTGTCTCCGCTTCT
1911	S	TCCAGGACTTTCCTCAGGTT
1965	S	TCCTGTCGTTTTTTGTCGTT
1968	S	TCGTCGCTGTTGTCGTTTCTT
2000	S	TCCATGACGTTCCTGCAGTTCCTGACGTT
2002	S	TCCACGACGTTTTCGACGTT
2006	S	TCGTCGTTTTGTCGTTTTGTCGTT
2007	S	TCGTCGTTGTCGTTTTGTCGTT
2014	S	TGTCGTTGTCGTTTTCTTGTCGTT
2041	S	CTGGTCTTTCTGGTTTTTTTCTGG
2054	S	TCCCGCGCGTTCCGCGCGTT
2055	S	TCCTGGCGGTCCTGGCGGTT
2135	S	TCGTCGTTTGTCGTTTTGTCGTT
2142	S	TCGCGTGCGTTTTGTCGTTTTGACGTT
2143	S	TTCGTCGTTTTGTCGTTTTGTCGTT
2166	S	ATAATTCGTCGTTCAAGCAAG
2186	S	TCGTCGCTGTCTCCGCTTCTTCTTGCC

[a]ODN are listed in numerical order, which is based on the synthesis run.

[b]Backbone details are abbreviated SOS (mixture of phosphorothioate and phosphodiester bonds within the same ODN), O (phosphodiester ODN), and S (phosphorothioate ODN).

[c]Nucleotide sequence of ODN. Potential stimulatory motifs are underlined.

Species

In order to take into consideration the genetic diversity of the species included in this study, peripheral blood was obtained, where possible from more than one breed. Age and gender were also taken into consideration when compiling appropriate donor panels for each species.

Peripheral blood from 11 domestic short hair and 2 domestic long hair cats was obtained from the Animal Resource Center at the University of Saskatchewan. Fourteen canine peripheral blood specimens provided by the Animal Resource Center included samples from 6 huskies, 4 beagles, 2 German shepherds, 1 golden retriever, and 1 collie.

Equine blood was kindly provided by the Western College of Veterinary Medicine at the University of Saskatchewan, and the panel of 8 samples was drawn from 3 standard bred horses, 2 quarter horses, 2 thoroughbreds, and 1 draft horse. Seven

porcine blood samples were provided by the Prairie Swine Center (Saskatoon, Saskatchewan, Canada), and all were the product of a terminal cross between a C15 sow and a Cana Boar (synthetic line from Pig Improvement Canada).

Blood from 6 male Suffolk sheep, 6 goats (2 females and 4 males), and 6 broiler chickens was used for screening. Splenocytes from 6 C57BL/6 mice and 6 *Sigmodon hispidus* cotton rats were used, and popliteal lymph nodes were taken from 9 New Zealand white rabbits.

Lymphocyte proliferation assay

Blood from all species was collected by venipuncture into Vacutainer tubes containing EDTA as an anticoagulant. Briefly, peripheral blood mononuclear cells (PBMC) from all species were isolated by centrifugation over Ficoll-Paque™ Plus (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) (Loehr et al., 2000). For chicken, feline, and canine samples, the centrifugation conditions were 2000 rpm for 30 minutes at 20°C in a Beckman CS-6R benchtop centrifuge (Beckman, Mississauga, Ontario, Canada). Pig, sheep, goat, and horse peripheral blood required centrifugation at 2500 rpm for 45 minutes at 20°C in order to isolate PBMC most effectively. Splenocytes were harvested from mice and cotton rats using standard protocols (Braun et al., 1998), and rabbit popliteal lymph node cells were extracted in the same manner.

Cells were subsequently washed three times with phosphate-buffered saline (PBS) (0.1 M NaH_2PO_4/Na_2HPO_4, 0.15 M NaCl, pH 7.4), followed by centrifugation, counted using a hemocytometer, and seeded at a concentration of 5×10^5 cells/ml in AIM-V medium (GIBCO-BRL, Burlington, Ontario, Canada) containing 2% (v/v) heat-inactivated fetal bovine serum (FBS) (GIBCO-BRL) and 50 μM 2-mercaptoethanol (BioRad, Mississauga, Ontario, Canada).

ODN were dispensed into 96-well round-bottom plates (Nalge-Nunc Int., Rochester, NY) in 10-μl volumes in triplicate. Cells were then added in a 200-μl volume to achieve a final ODN concentration of 4 μg/ml in the presence of 1×10^5 cells per well. The ODN and cell concentrations were chosen on the basis of our previous experience with bovine PBMC, which showed that this dose provided reliable levels of proliferation while minimizing nonspecific proliferation. Controls consisted of 6 wells per plate with cells only. After 72 hours of incubation at 37°C in a humidified 5% CO_2 incubator, the cells were pulsed with 20 μl of a 20 μCi/ml solution of methyl-^{3}H-thymidine (Amersham Pharmacia Biotech) for 18 hours. The cells were then harvested onto glass fiber filtermats (Skatron, Liebyen, Norway), and radiolabel uptake was determined by liquid scintillography using a β-counter (Beckman). Stimulation indices (SI) were calculated by the standard method of dividing the cpm in test triplicates by those of the unstimulated control cells (Loehr et al., 2000).

Immunization of sheep

Five-month-old Suffolk sheep were immunized intradermally with a truncated secreted version of glycoprotein B (tgB) (Li et al., 1996), which is one of the protective antigens of bovine herpesvirus-1 (BHV-1). The sheep weighed approximately 40 kg, and they were randomly allocated to 5 different groups of 5 animals each. Glycoprotein B was formulated at 20 mg per dose in phosphate-buffered saline (PBS) alone or in combination with 0.8 mg, 4 mg, or 20 mg of ODN 2135. One control group was immunized with PBS and another group with tgB in VSA3, an oil-based adjuvant (van Drunen Littel-van den Hurk et al., 1994). The volume of each vaccine formulation was 500 μl. Four weeks later, they received a second immunization. The sheep were bled at the time of immunization, as well as at different times after each immunization. The experiment was carried out according to the guidelines provided by the Canadian Council on Animal Care.

Enzyme-linked immunosorbent assays (ELISA)

Polystyrene microtiter plates (Immulon 2) (Dynatech Laboratories, Gaithersburg, MD) were coated with 0.05 μg affinity-purified tgB (Li et al., 1996; Loehr et al., 2000) per well and incubated with serially diluted ovine sera. Alkaline phosphatase-conjugated rabbit antisheep IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a dilution of 1:10,000 was used as the detecting antibody. The reaction was visualized with p-nitrophenyl phosphate (Sigma Chemical Co., St Louis, MO).

Neutralization test

The neutralization titers in the bovine sera were determined as previously described and expressed as the reciprocal of the highest dilution of antibody that caused a 50% reduction of plaques relative to the virus control (van Drunen Littel-van den Hurk et al., 1994).

Statistical analyses

Data were analyzed with the aid of a statistical software program (Graphpad™ Prism). ELISA and neutralization titers were expressed as the $\log_{10}$ reciprocal of the end point. Differences between the vaccine groups were examined by performing a one-way ANOVA.

RESULTS

Identification of immunostimulatory CpG motif

Depending on the species tested, between 6 and 16 ODN induced lymphocyte proliferation (Tables 1 and 2 and Figs. 1 and 2), and for most species, there was a broad similarity in the profile of the stimulatory ODN. The predominant feature of these ODN was the presence of the GTCGTT motif (Table 1), so there appeared to be little evidence of divergence among the species in terms of actual motif recognition and no apparent differences in terms of the other requirements for ODN stimulation, such as the nature and degree of spacing between motifs or the requirements for particular 5′ bases preceding the motifs. Also, there was no apparent variation in the stimulatory effect of ODN based on age, gender, or breed. Generally, the ODN with three GTCGTT motifs (2006, 2007, 2014, 2135, 2143) had a strong and constant stimulatory effect, whereas ODN with one or two GTCGTT motifs (1965, 1968, 2166) did not activate the immune cells as consistently. Interestingly, ODN 2142 with

TABLE 2. REACTIVITY PATTERNS OF INDIVIDUAL SPECIES TO EACH ODN

ODN[a]	1585	2001	2037	2038	2039	2040	2079	2080	2082	1760	1826	2000	2002	1965	1968	2006	2007	2014	2135	2142	2143	2166	1835	1841	1842	1911	2041	2054	2095	2186
Sheep																														
Goat																														
Horse																														
Pig																														
Dog																														
Cat																														
Chicken																														
Cotton rat																														
Rabbit																														
Mouse																														

[a]Compartments are shaded if the mean proliferative response of a particular ODN and species was at least 50% of the value for the maximally stimulatory ODN for that species.

one GTCGTT, one GACGTT, and one TGCGTT motif, was stimulatory to immune cells from all but 2 of the species, and ODN 2186 with one GTCGCT motif stimulated cells from 7 species. The motif optimal for stimulating murine cells (GACGTT) was also able to induce proliferative responses in rabbit lymphocytes but not in the cells from the other species tested. Among the ODN with a native phosphodiester backbone, ODN 2079, 2080, and sometimes 2082 had a stimulatory effect, which may be due to the poly-C tail, specifically for ODN 2079 and 2082, which do not have the GTCGTT motif.

Adjuvanticity of immunostimulatory ODN in sheep

To determine if immunostimulatory ODN can be used as an adjuvant in a species of veterinary importance, the immune responses induced to BHV-1 tgB formulated either with CpG or with VSA3, an oil-based adjuvant (van Drunen Littel-van den Hurk et al., 1994), were determined in sheep. ODN 2135 was selected for this experiment, as it induced the strongest proliferation in the PBMC from sheep as well as a strong response in cells from all but one of the other species tested. After one immunization, the sheep immunized with tgB and ODN 2135 or VSA3 developed low antibody titers, whereas the animals immunized with tgB in PBS did not have detectable antibody levels (Fig. 3A). Although all titers increased after the second immunization, the responses induced by tgB in the presence of ODN 2135 or VSA3 were still significantly higher than those induced by tgB in PBS ($p < 0.05$), which confirms the adjuvant effect of ODN 2135. Although there was no difference between 0.8 mg of ODN 2135, 4 mg of ODN 2135, and VSA3, 20 mg of ODN2135 induced significantly higher titers ($p < 0.05$).

To assess the biologic relevance of the antibody responses elicited with the different tgB formulations, virus neutralization (VN) titers were determined 2 weeks after the second immunization. The VN titers were similar in the groups immunized with tgB formulated with 4 mg of ODN 2135 or VSA3, whereas the VN titers were higher in the group that received tgB with 20 mg of ODN 2135, which confirmed that the tgB-specific antibodies induced with CpG ODN as an adjuvant have equivalent neutralizing capacity to the antibodies elicited with a conventional oil-based adjuvant (Fig. 3B).

DISCUSSION

Immunostimulation by CpG DNA represents a novel immunologic process of nonself recognition by vertebrates that is being capitalized on for practical applications in human medicine. The need for safer and more efficacious adjuvants and immunotherapies for humans and animals, as well as the application of animal models to human disease, makes CpG an attractive option for further investigation in veterinary species. Here, the scope for future research within this field has been significantly broadened with the identification of biologically active CpG motifs and ODN for a number of important veterinary and laboratory species.

Although it is difficult to make concrete comparisons between species, especially given the differences in absolute *in vitro* responsiveness to the ODN panel and a lack of differences between most of the stimulatory ODN, some outstanding features were detected. In all species, the GTCGTT motif appeared to have stimulatory potential, and the number and sequence context of this motif also influenced the biologic activity of the ODN. ODN without CpG motifs were nonstimulatory in the proliferation assays, whereas there were diverse levels in responsiveness to CpG ODN. The stimulatory capacity of the ODN was dependent not only on sequence but also on the

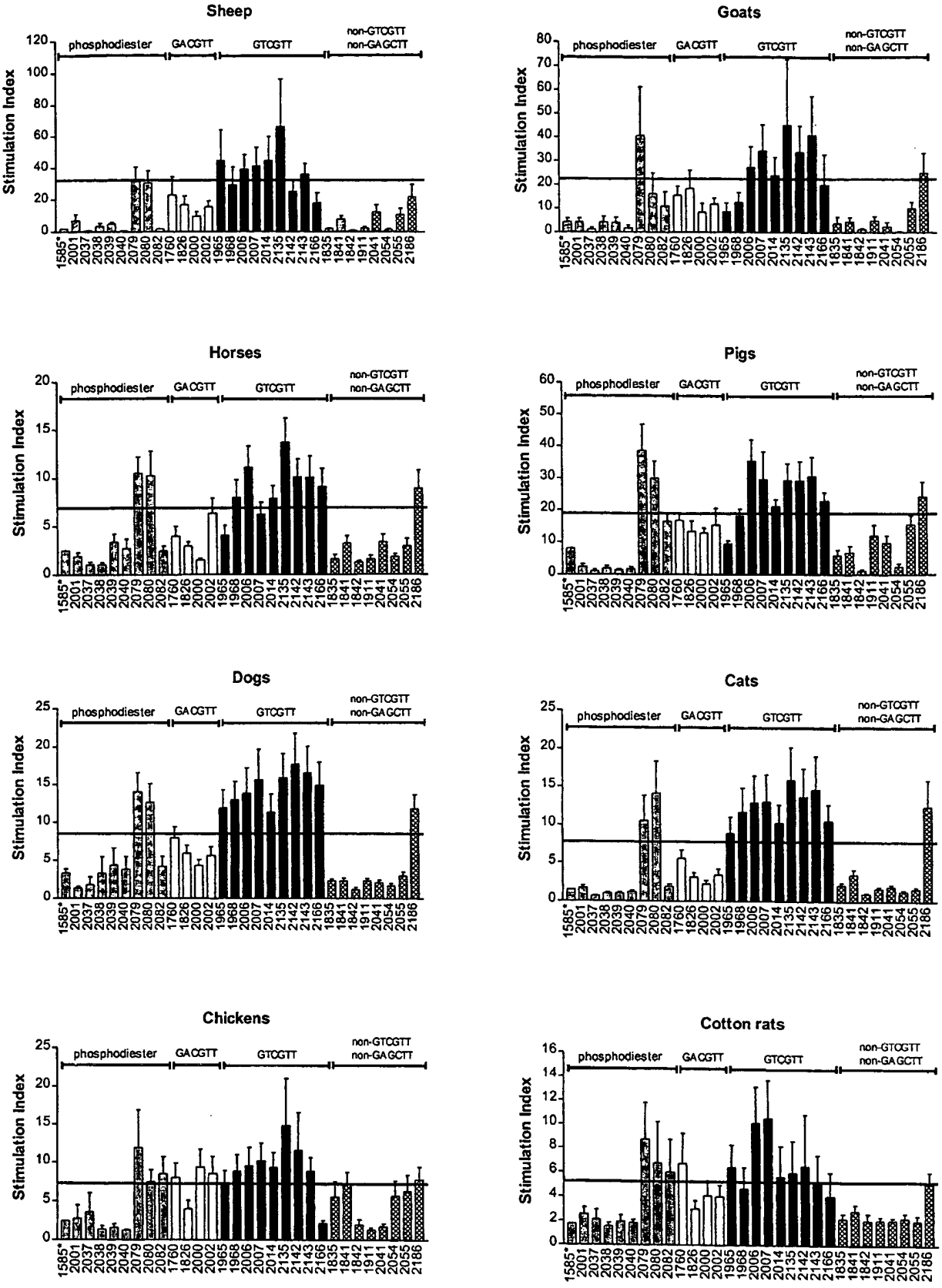

FIG. 1. Proliferation of PBMC in peripheral blood from 6 sheep, 6 goats, 8 horses, 7 pigs, 14 dogs, 13 cats, and 6 chickens and splenocytes from 6 cotton rats in response to incubation with different ODN. The results represent the average of triplicate wells and are expressed as mean SI ± SEM for each ODN. The horizontal line represents 50% of the SI achieved with the maximally stimulatory ODN. All ODN except the ones indicated by phosphodiester are phosphorothioate modified. *Mixture of phosphorothioate and phosphodiester bonds.

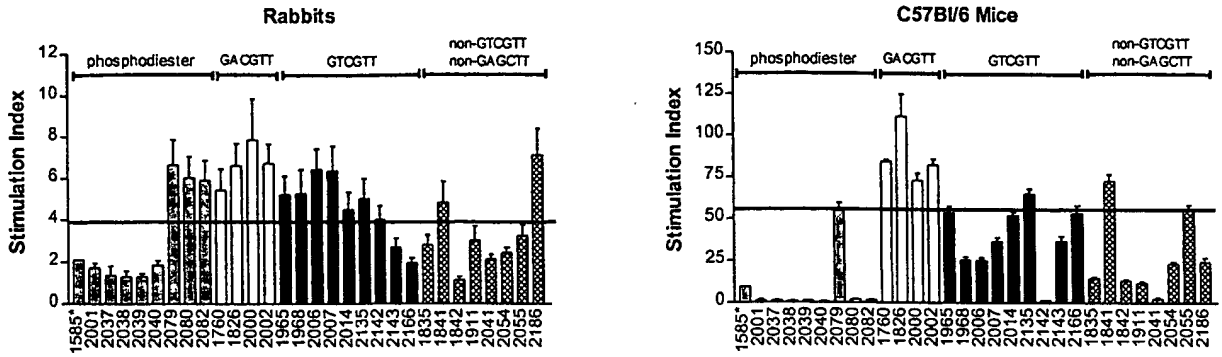

FIG. 2. Proliferation of PBMC in peripheral blood from popliteal lymph nodes from 9 rabbits and splenocytes from 6 C57BL/6 mice in response to incubation with different ODN. The results represent the average of triplicate wells and are expressed as mean SI ± SEM for each ODN. The horizontal line represents 50% of the SI achieved with the maximally stimulatory ODN. All ODN except the ones indicated by phosphodiester are phosphorothioate modified. *Mixture of phosphorothioate and phosphodiester bonds.

chemical nature of the backbone. In general, ODN with a native phosphodiester backbone were nonstimulatory, with the exception of ODN 2079, 2080, and 2082. Interestingly, both ODN 2079 and 2080, which contain only one motif followed by a poly-C tail, have been shown previously to stimulate human immune cells (Hartmann et al., 2000). In this case, the poly-C component may permit enhanced ODN uptake or stabilize the ODN against degradation, thus accounting for the fact that a GACGTT motif in 2079 could stimulate proliferative responses as well as ODN 2080, which contains the optimal GTCGTT motif. Consistent with previous observations (Hartmann et al., 2000), ODN 2082, which has a GCCGTT motif, generally did not have a strong stimulatory effect on PBMC.

Previous studies have identified optimal ODN sequences for stimulating murine, primate, and monkey immune cells (Chace et al., 1997; Jones et al., 1999; Davis et al., 2000; Hartmann et al., 2000). Furthermore, motifs that activate bovine, feline, and canine cells *in vitro* have also been identified (Brown et al., 1998; Pontarollo et al., 2001, in press; C.M. Wernette et al., unpublished observations). An ODN with a TpC dinucleotide at the 5′-end followed by copies of the optimal CpG motif (GTCGTT), separated by TpT dinucleotides, appears to be optimal for stimulation of human, rhesus monkey, chimpanzee, and bovine lymphocytes (Jones et al., 1999; Davis et al., 2000; Hartmann et al., 2000; Pontarollo et al., 2001, in press). Recent evidence has suggested that the lower and higher vertebrates recognize different motifs, as immune responses in fish were not enhanced using CpG ODN that have adjuvant capacity in mice (Kanellos et al., 1999). However, subsequent studies have shown that fish, like the species described in this report, also respond to the GTCGTT motif (H. Davis, personal communication). Including the data presented here, CpG motifs have been identified for 15 species. Immune cells from all these species were stimulated by the GTCGTT motif, indicating that CpG motif recognition is an ancient evolutionary adaptation that is highly conserved in a broad variety of species across three vertebrate phyla. The only apparent exceptions appear to be laboratory mice and rabbits, which also recognize the GACGTT motif. This phenomenon may be due to the fact that the strains of those species screened for CpG ODN reactivity were inbred. It should be noted that the other laboratory species screened in

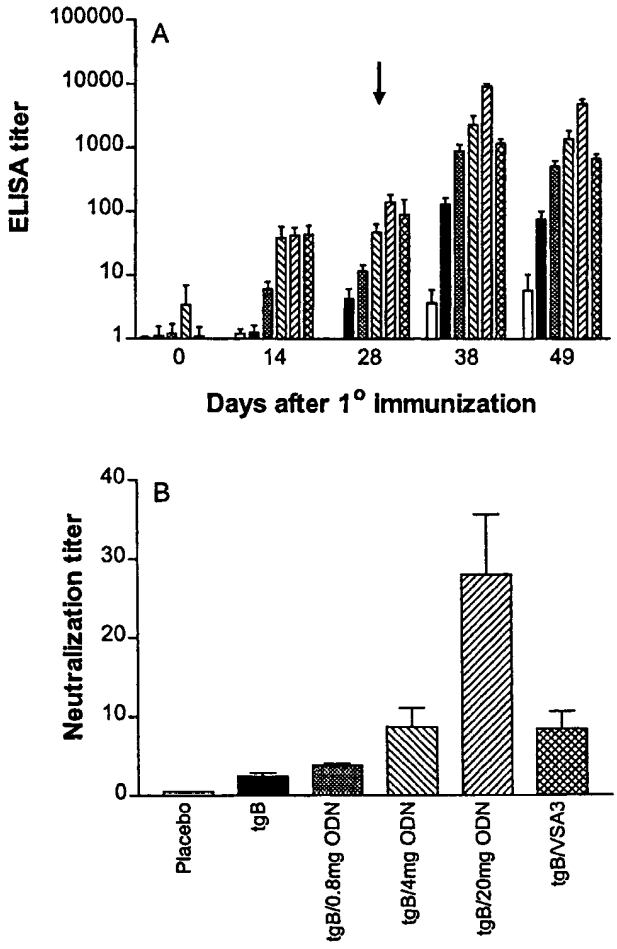

FIG. 3. Serum antibody responses in sheep immunized twice with PBS (□), tgB in PBS (■), tgB with 0.8 mg ODN 2135 (■), tgB with 4 mg ODN 2135 (▨), tgB with 20 mg ODN 2135 (■), or tgB with VSA3 (▩). (A) Geometric mean tgB-specific ELISA titers, expressed as the reciprocal of the highest dilution resulting in a reading of 2 SD above the control value. The arrow indicates the time point of the secondary immunization on day 28. (B) Geometric mean virus-neutralizing antibody titers 2 weeks after the second immunization and expressed as a 50% end point using 100 pfu of BHV-1. Error bars show the SD of the means.

this study was an outbred cotton rat strain, and the observed pattern of reactivity was the same as for the other outbred species used in this study. In addition to further defining the mode of action of CpG ODN in veterinary species at, for example, the cytokine level, future work may elucidate whether inbreeding has an impact on CpG recognition. Our data are supported by the recent identification of Toll-like receptor-9 (TLR-9) as a CpG recognition molecule (Hemmi et al., 2000), which suggests a high degree of evolutionary conservation in the recognition mechanism, regardless of what is the actual receptor or receptor complex. It will now be possible to compare TLR-9 sequences in the various species and define the similarity with the TLR-9 sequences in mice and humans.

Studies in mice, monkeys, and primates suggest that ODN-induced *in vitro* proliferation correlates well with the capacity to enhance immune responses (Lipford et al., 1997; Chu et al., 1997; Davis et al., 1998, 2000; Jones et al., 1999). In orangutans, which weigh 5–15 kg, a little less than the sheep used in this study, ODN 2006 at a dose between 30 μg and 1 mg was found to enhance immune responses to HBsAg (Davis et al., 2000). Although further experimentation would be required to fully determine the quality of the active ODN identified in this study, the ODN that induced the strongest *in vitro* proliferation of ovine PBMC also acted as an efficient adjuvant for a recombinant protein vaccine *in vivo* in sheep. At a dose of 0.8–4 mg, the adjuvant effect of ODN 2135 was as strong as that of an oil-based adjuvant that has been used frequently with recombinant protein vaccines (van Drunen Littel-van den Hurk et al., 1994). At 20 mg, the adjuvant capacity of ODN 2135 was even stronger. As observed for ODN 2006 in orangutans (Davis et al., 2000), there was a trend for higher titers at the higher dose of ODN 2135 in sheep. Consequently, the data presented here confirm the adjuvanticity of CpG ODN in a veterinary species.

Not only do these data have relevance to veterinary medicine, but they may also allow the properties of CpG to be tested in animal models of human disease, such as respiratory syncytial virus, rabies, virus, and mycobacterial infections (Sharma and Woldehiwet, 1991; Baltazar et al., 1992; Piazza et al., 1993; Burrells et al., 1995). Furthermore, the potential of using large animal models of human disease will allow studies on CpG that are difficult or impossible to perform in mice, such as the impact of CpG ODN on regional immunity and toxicologic effects of CpG at clinically relevant doses.

In conclusion, this is the first report describing active CpG motifs for a number of important livestock and companion animals. Additionally, based on our data and those of others, it is becoming apparent that CpG motif recognition is highly conserved in a broad variety of species. The data presented in this report widen the scope for CpG technology and will allow systematic testing of the efficacy of this promising new pharmaceutical in many new applications.

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REFERENCES

BALLAS, Z.K., RASMUSSEN, W.L., and KRIEG, A.M. (1996). Induction of natural killer activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J. Immunol. **157,** 1840–1845.

BALTAZAR, S.R., ARTOIS, M., and BLANCOU, J. (1992). Experimental infection of sheep with a rabies virus of canine origin: Study of the pathogenicity for that species. Rev. Sci. Technol. **11,** 829–836.

BRAUN, R., BABIUK, L.A., and VAN DRUNEN LITTEL-VAN DEN HURK, S. (1998). Compatibility of plasmids expressing different antigens in a single DNA vaccine formulation. J. Gen. Virol. **79,** 2965–2970.

BROWN, W.C., ESTES, D.M., CHANTLER, S.E., KEGERREIS, K.A., and SUAREZ, C.E. (1998). DNA and a CpG oligonucleotide derived from *Babesia bovis* are mitogenic for bovine B cells. Infect. Immun. **66,** 5423–5432.

BURRELLS, C., INGLIS, N.F., DAVIES, R.C., and SHARP, J.M. (1995). Detection of specific T cell reactivity in sheep infected with *Mycobacterium avium* subspecies *silvaticum* and *paratuberculosis* using two defined mycobacterial antigens. Vet. Immunol. Immunopathol. **45,** 311–320.

CARDON, L.R., BURGE, C., CLAYDON, D.A., and KARLIN, S. (1994). Pervasive CpG suppression in animal mitochondrial genomes. Proc. Natl. Acad. Sci. USA **91,** 3799–3803.

CARPENTIER, A.F., CHEN, L., MALTONI, F., and DELATTRE, J.Y. (1999). Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mice. Cancer Res. **59,** 5429–5432.

CHACE, J.H., HOOKER, N.A., MILDENSTEIN, K.L., KRIEG, A.M., and CHOWDERY, J.S. (1997). Bacterial DNA-induced NK cell IFNγ production is dependent on macrophage secretion of IL-12. Clin. Immunol. Immunopathol. **84,** 185–193.

CHU, R.S., TARGONI, O.S., KRIEG, A.M., LEHMAN, P.V., and HARDING, C.V. (1997). CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J. Exp. Med. **186,** 1623–1631.

COWDERY, J.S., CHACE, J.H., YI, A.K., and KRIEG, A.M. (1996). Bacterial DNA induces NK cells to produce IFN-γ *in vivo* and increases the toxicity of lipopolysaccharides. J. Immunol. **156,** 4570–4575.

DAVIS, H.L., SUPARTO, I., WEERATNA, R., JUMINTARTO, ISKANDRIATI, D., CHAMZAH, S., MA'RUF, A., NENTE, C., PAWITRI, D., KRIEG, A.M., HERIYANTO, SMITS, W., and SAJUTHI, D. (2000). CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine **18,** 1920–1924.

DAVIS, H.L., WEERATNA, R., WALDSCHMIDT, T.J., TYGRETT, L., SCHORR, J., and KRIEG, A.M. (1998). CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J. Immunol. **160,** 870–876.

HARTMANN, G., WEERATNA, R., BALLAS, Z.K., PAYETTE, P., SUPARTO, I., RASMUSSEN, W.L., WALDSCHMIDT, M., SAJUTHI, D., PURCELLS, R.H., DAVIS, H.L., and KRIEG, A.M. (2000). Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses *in vitro* and *in vivo*. J. Immunol. **164,** 1617–1624.

HEMMI, H., TAKEUCHI, O., KAWAI, T., KAISHO, T., SATO, S., SANJO, H., MATSUMOTO, M., HOSHINO, K., WAGNER, H.,

TAKEDA, K., and AKIRA, S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature **408,** 740–745.

JONES, T.R., OBALDIA, N., III, GRAMZINSKI, R.A., CHAROENVIT, Y., KOLODNY, N., KITOV, S., DAVIS, H.L., KRIEG, A.M., and HOFFMAN, S.L. (1999). Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in Aotus monkeys. Vaccine **17,** 3065–3071.

KANELLOS, T.S., SYLVESTER, I.D., BUTLER, V.L., AMBALI, A.G., PARTIDOS, C.D., HAMBLIN, A.S., and RUSSEL, P.H. (1999). Mammalian granulocyte-macrophage colony-stimulating factor and some CpG motifs have an effect on the immunogenicity of DNA and subunit vaccines in fish. Immunology **96,** 507–510.

KLINE, J.N., BUSINGA, T.R., WALDSCHMIDT, T.J., WEINSTOCK, J.V., and KRIEG, A.M. (1998). Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. J. Immunol. **15,** 2555–2559.

KLINMAN, D.M. (1998). Therapeutic applications of CpG-containing oligodeoxynucleotides. Antisense Nucleic Acid Drug Dev. **8,** 181–184.

KRIEG, A.M. (2000). The role of CpG motifs in innate immunity. Curr. Opin. Immunol. **12,** 35–43.

KRIEG, A.M., YI, A.K., MATSON, S., WALDSCHMIT, T.J., BISHOP, G.A., TEASDALE, R., KORETZKY, G.A., and KLINMAN, D.M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature **374,** 546–549.

KRIEG, A.M., YI, A.K., SCHORR, J., and DAVIS, H.L. (1998). The role of CpG dinucleotides in DNA vaccines. Trends Microbiol. **6,** 23–27.

LI, Y., VAN DRUNEN LITTEL-VAN DEN HURK, S., LIANG, X., and BABIUK, L.A. (1996). Production and characterization of bovine herpesvirus-1 glycoprotein ectodomain derivatives in an hsp70A gene promoter-based expression system. Arch. Virol. **141,** 2019–2029.

LIPFORD, G.B., BAUER, M., BLANK, C., REITER, R., WAGNER, H., and HEEG, K. (1997). CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: A new class of vaccine adjuvants. Eur. J. Immunol. **27,** 2340–2344.

LOEHR, B.I., WILLSON, P., BABIUK, L.A., and VAN DRUNEN LITTEL-VAN DEN HURK, S. (2000). Gene gun-mediated DNA immunization primes development of mucosal immunity against bovine herpesvirus 1 in cattle. J. Virol. **74,** 6077–6086.

MAGONE, M.T., CHAN, C.C., BECK, L., WHITCUP, S.M., and RAZ, E. (2000). Systemic or mucosal administration of immunostimulatory DNA inhibits early and late phases of murine allergic conjunctivitis. Eur. J. Immunol. **30,** 1841–1850.

MOLDOVENU, G.J., LOVE-HOMAN, L., HUANG, W.Q., and KRIEG, A.M. (1998). CpG DNA, a novel immune enhancer for systemic and mucosal immunisation with influenza virus. Vaccine **16,** 1216–1224.

PIAZZA, F.M., JOHNSON, S.A., DARNELL, M.E., PORTER, D.D., HEMMING, V.G., and PRINCE, G.A. (1993). Bovine respiratory syncytial virus protects cotton rats against human respiratory syncytial virus infection. J. Virol. **67,** 1503–1510.

PONTAROLLO, R.A., RANKIN, R., BABIUK, L.A., GODSON, D.L., GRIEBEL, P.J., HECKER, R., KRIEG, A.M. and VAN DRUNEN LITTEL-VAN DEN HURK, S. (2001). VIDO's Journal Series No. 293, in press.

SHARMA, R., and WOLDEHIWET, Z. (1991). Immune responses of lambs experimentally infected with bovine respiratory syncytial virus and *Pasteurella haemolytica*. J. Comp. Pathol. **105,** 157–166.

SPARWASSER, T., KOCH, E.S., VABULAS, R.M., HEEG, K., LIPFORD, G.B., ELLWART, J.W., and WAGNER, H. (1998). Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur. J. Immunol. **28,** 2045–2054.

SUN, S., ZHANG, X., TOUGH, D.F., and SPRENT, J. (1998). Type I interferon-mediated stimulation of T cells by CpG DNA. J. Exp. Med. **188,** 2335–2342.

VAN DRUNEN LITTEL-VAN DEN HURK, S., VAN DONKERSGOED, J., KOWALSKI, J., VAN DEN HURK, J.V., BABIUK, L.A., and ZAMB, T.J. (1994). A subunit gIV vaccine, produced by transected mammalian cells in culture, induces mucosal immunity in cattle. Vaccine **12,** 1295–1302.

WEERATNA, R., McCLUSKIE, M.J., YU, X., and DAVIS, H.L. (2000). CpG DNA induces stronger immune responses with less toxicity than other adjuvants. Vaccine **18,** 1755–1762.

WOOLDRIDGE, J.E., BALLAS, Z., KRIEG, A.M., and WEINER, G.J. (1997). Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. Blood **89,** 2994–2998.

YI, A.K., CHANG, M., PECKHAM, D.W., KRIEG, A.M., and ASHMAN, R.F. (1998). CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. J. Immunol. **160,** 5898–5906.

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Positive and negative regulation of interleukin-12 gene expression

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ABSTRACT. Interleukin-12 (IL-12) is a pivotal cytokine representing the link between the cellular and humoral branches of an effective host immune defense apparatus. IL-12 is a heterodimer produced by phagocytic, B, dendritic, and possibly other accessory cells in both innate and adaptive immune responses. It is a key factor in the induction of T cell-dependent and independent activation of macrophages, generation of T helper type 1 (Th1) and cytotoxic T cells, suppression of IgG1 and IgE production, induction of organ-specific autoimmunity, and resistance to bacterial and parasitic infections [1]. IL-12 has a powerful anti-tumor and anti-metastatic activity against many murine tumors [2-5] as well as human tumors [6-17]. The genes encoding the two heterologous chains of IL-12, p40 and p35 are located on different human chromosomes. Together, p40 and p35 form the biologically active IL-12. Their expressions are highly cordinated during an effective immune response. However, under some pathological conditions, IL-12 is under- or overexpressed, resulting either in a lack of resistance to microbial infection and to uncontrolled tumor growth, or in destructive inflammation, respectively. A transient or irreversible dysregulation of IL-12 production may reflect a pathogen/tumor cell-induced disruption in the highly coordinated expression of p40 and p35. The understanding of the molecular mechanisms governing the expression of IL-12 p40 and p35 genes in the context of interactions between pathogens and the immune system is essential in efforts aimed at designing therapeutic strategies to treat infectious and malignant diseases.

Keywords : IL-12, gene expression

CELLULAR INDUCTION OF IL-12.

IL-12 was originally discovered and characterized as a product of the EBV-transformed lymphoblastoid B cell lines RPMI-8866, ADP, and NC37 [18,19]. Phagocytic cells, however, have been subsequently and firmly established as a major source of IL-12 production by many *in vitro* and in vivo studies in infectious disease models [20]. PBMC or purified monocytes produce high levels of IL-12 p40 and p70 when stimulated by bacteria, such as heat-fixed *Staphylococcus aureus* (SAC) or *Staphylococcus* extracts, or by bacterial products such as LPS [21]. The producer cells within PBMC are mostly monocytes and other MHC class II-positive cells, possibly dendritic cells [21]. The production of IL-12 by phagocytic cells, and at least in part, by dendritic cells, is induced by a variety of mechanisms that reflect the role played by IL-12 in inflammation and immunity.

These mechanisms are either T cell-independent or -dependent. The T cell-independent mechanisms are important for the proinflammatory and immunoregulatory role of IL-12 at the interface of innate resistance and adaptive immunity. These mechanisms are exemplified by the induction of IL-12 by infectious agents such as bacteria, both protozoan and metazoan parasites, fungi, and viruses as well as their products, of which LPS and bacterial DNA are the most typical examples [21, 22].

During inflammation, however, an important mechanism of infection-independent induction of IL-12 and other cytokines is represented by interaction of adhesion molecules with substrates of inflammatory origin, exemplified by the interaction of CD44 adhesion molecules with low molecular weight hyaluronan [23]. The T cell-dependent mechanism of IL-12 production is dependent on the ability of CD40 ligand (CD40L) expressed on activated T cells to interact with CD40 receptor on the surface of monocyte/macrophages and dendritic cells [24, 25]. The T cell-dependent mechanisms of IL-12 induction play an important role in the T cell immunoregulatory role of IL-12 and in particular in the maintenance of Th1 responses.

MODULATION OF IL-12 PRODUCTION AT THE CELLULAR LEVEL.

IL-12 production during an immune reaction is tightly modulated by both positive and negative feedback signals. The positive feedback regulation is exemplified by IFN-γ which is induced by IL-12 initially from NK and T cells and which in turn potently enhances the ability of monocytes and polymorphonuclear cells (PMN) to produce IL-12 [26, 27]. The priming effect of IFN-γ for augmented IL-12 production may represent a mechanism by which IL-12-induced Th1 responses are maintained *in vivo*. This effect is materialized only after extended treatment of monocytes with IFN-γ (>12 h) prior to stimulation with bacterial products such as LPS, suggesting that it may be secondary to an activation/maturation/differentiation effect induced by IFN-γ. The positive feedback amplification of IL-12 production mediated by IFN-γ is a potentially dangerous mechanism leading to uncontrolled cytokine production and possibly shock.

There are also effective mechanisms that down-regulate IL-12 production and the responsiveness of T and NK cells to IL-12. The Th2 cytokine IL-10 is a potent inhibitor of IL-12 production from phagocytic cells; the ability of IL-10 to suppress production of IFN-γ and other Th1 cytokines is due primarily to its inhibition of IL-12 production from APC and to its inhibition of expression of other costimulatory surface molecules (e.g. B7) and soluble cytokines (e.g., TNF-α, IL-1β) (28-30). Another powerful inhibitor of IL-12 production is TGF-β, a product of neoplastic transformation [31].

An important mechanism of cross-talk between innate resistance and adaptive immunity is the ability of complement components and immunoglobulins to react with complements or Fc receptors (FcR) on the effector cells of innate resistance, modulating their functions and cytokine production. The first indication that IL-12 production is also regulated by those interactions was provided by the observation that measles virus induces a profound depression of Th1 responses following infection, in part by interacting with its receptor on phagocytic cells, the CD46 molecule, to inhibit IL-12 production [32]. CD46, or membrane cofactor protein, is a binding site for C3b and C4b. By binding to CD46, both polymeric C3b and anti-CD46 antibodies can inhibit IL-12 production, suggesting that measles virus may utilize a physiological mechanism of selectively downregulating IL-12 through activated complements in its induction of immunosuppression [32]. HIV activates the complement system in human plasma and may interact with complement regulatory molecules for its own advantage: enhancement of infectivity, follicular localization, and broadening of its host range while displaying an intrinsic resistance against the lytic action of human complement. Whether there is a direct linkage between HIV-activated complement system and the recently documented suppression of IL-12 production in HIV-infected individuals [29, 33] remains to be established.

Immunocomplexes occur in a number of chronic human diseases in which autoimmune symptoms are not the main feature, but in which sufficient Th1-type immune responses are critical and an impairment of IL-12 secretion may be responsible for the disease progression, such as in HIV-infection, tuberculosis, and malignant processes [34, 35]. The ability to trigger monocyte/macrophage receptors and selectively suppress IL-12 production was extended to the interaction of C3bi with complement receptor 3 (CD11b) and to the interaction of immunocomplexes with FcR or scavenger receptors [36-38]. CD11b and FcR have also been shown to interact with each other and synergize in functional activation of the effector cells, suggesting possible converging signal transduction pathways [39-42]. The FcR-mediated inhibition of IL-12 is interesting because cross-linking of these receptors is also a potent stimulus for the induction of other proinflammatory cytokines [43-51]. This phenomenon may be related to the notion that aggregating FcR may result in both positive and negative signals, depending on the types of FcR that are interacting. For instance, interaction between FcR containing immunoreceptor tyrosine-based activating motif (ITAM) and FcR containing immunoreceptor tyrosine-based inhibitory motif (ITIM) results in negative cooperation [52]. Calcium fluxes and the production of prostaglandin E2 are implicated in FcR-mediated inhibition of IL-12 production. IL-10 and TNF-α are partially responsible for this inhibition since it has been demonstrated that the use of anti-IL-10 and anti-TNF-α antibodies resulted in an incomplete reversal of the suppression of IL-12 production mediated by immunocomplexes or FcR-ligation [38]. Moreover, in IL-10-deficient mice, the FcR-mediated down-regulation of IL-12 was also observed (D. Mosser, personal communication). This cytokine down-regulation after receptor

ligation may contribute to the transient nature of IL-12 in plasma during bacteremia, and it may result in diminished IL-12 production in an immune host due to the rapid clearance of IgG-opsonized bacteria. The receptor-mediated inhibition of IL-12 induction may also be exploited by intracellular pathogens of macrophages to down-regulate IL-12 production and suppress or delay the development of cell-mediated immunity.

MOLECULAR MECHANISM OF IFN-γ PRIMING FOR IL-12 P40 GENE EXPRESSION.

The analysis of the molecular control of IL-12 production is complicated by the need to study the coordinated expression of both p40 and p35 genes. The p40 gene is highly inducible and expressed only in IL-12-producing cells, compared with that of the more ubiquitously expressed p35 gene. We examined simultaneously nuclear transcription, steady-state mRNA and secreted protein levels of IL-12, and established that the human IL-12 p40 gene is primarily regulated at the transcriptional level by IFN-γ and LPS in monocytic cells [53]. Both the human and mouse IL-12 p40 promoters have been cloned [53, 54]. The 3.3 kb human p40 promoter, when linked to a luciferase reporter gene, transiently or stably transfected into various IL-12-producing and non-producing cell lines, largely recapitulated the cell specificity of the endogenous p40 gene, i.e., it is constitutively active in EBV-transformed B cell lines (e.g., RPMI-8866, CESS), and inducible by LPS in myeloid cell lines (e.g., THP-1 and RAW264.7), but inactive in T cell lines (e.g., Molt-13 and Jurkat). Moreover, this promoter construct responds to IFN-γ-priming in monocytic cells much like the endogenous p40 gene, suggesting that it contains sufficient sequence elements to reconstitute the *in vivo* response.

A detailed functional dissection of the human p40 promoter in monocytic and EBV-B cell lines has identified two critical *cis*-elements involved in the regulation of the p40 gene transcription by LPS and IFN-γ. An ets site at -211/-206 (TTTCCT) and an "NFκB half site" at -117/-107 (TGAAATTCCCC). The ets site and its surrounding sequences (-292/-196) interacts with a very large complex named F1 which is induced by either LPS or IFN-γ and which is composed of ets-2, IRF-1, NFκB c-Rel, and a novel, ets-2-related protein with a molecular mass of 109 kDa (thus named GLp109 for its inducibility by LPS and IFN-γ). 5' deletion of 27 bp of this region (-265/-196) revealed a second complex named F2 which is more responsive to IFN-γ than to LPS and which does not appear to be related to any known IFN-γ-inducible factor or to any members of the NFκB family. The "NFκB half site" binds p50/p65 and p50/c-Rel heterodimers induced by LPS. Both elements are essential since deletion or mutation of specific nucleotides within these sites abolish the p40 promoter activity. Cotransfection experiments with various combinations of expression vectors for NFκB p65, p50, c-Rel, and ets-2 demonstrated that ets-2 and c-Rel synergistically activate the transfected p40 promoter in both IL-12 p40-expressing cells (RPMI-8866, RAW264.7), and non-expressing cells such as Bjab [EBV(-) B cell line] and Jurkat (T cell line) [55], strongly suggesting that c-Rel and ets-2 are the major transcription factors necessary and sufficient to direct the cell type-specific expression of the p40 gene.

The presence of IRF-1 in the F1 complex, thereby its involvement in the regulation of p40 gene expression, is strongly suggested by the observation of deficient IL-12 production and impaired Th-1 responses in IRF-1-null mice [55]. Another gene that is induced by IFN-γ is the interferon consensus binding protein (ICSBP) which belongs to the IRF family of transcription factors but which is expressed exclusively in cells of the immune system. Mice with a disrupted ICSBP gene are selectively deficient in IL-12 p40 gene expression and highly susceptible to infection with intracellular pathogens such as *L. monocytogenes* and *T. gondii* [56, 57]. The ets site in the p40 promoter bears strong resemblance to the ISRE that ICSBP interacts with.. Our functional studies indicate that ICSBP can act through this site and synergize specifically with IRF-1 to activate the human p40 promoter in RAW 264.7 cells [58].

MOLECULAR REGULATION OF IL-12 P35 GENE EXPRESSION.

Analysis of IL-12 p35 gene expression is hindered not only by its ubiquity, but also by its low activity and inducibility, until the priming effect of IFN-γ was discovered [27, 53]. Our comparative studies with cycloheximide (CHX) revealed striking differences in mRNA regulation between the p40 and p35 genes, i.e. the *S. aureus*- or LPS-induced p40 mRNA was abrogated when cells were pretreated with CHX, suggesting that the regulation of IL-12 p40 gene requires the induction of a CHX-sensitive transcription factor(s). In contrast, IL-12 p35 mRNA was "superinduced" by CHX, indicating that the activation of the p35 mRNA requires only a presynthesized activator(s) that can be induced either by *S. aureus* or LPS at the posttranslational level [59].

The genomic structure of both mouse and human IL-12 p35 genes has been determined. In both species, the p35 gene consists of either 8 [60] or 7 [61, 62] exons, as analyzed by primer extension [61] or by 5' RACE [60]. This genomic structure is further suggested by the isolation of murine p35 cDNA with alternative 5ſ untranslated regions [60, 63]. The nucleotide sequence of human p35 cDNA

isolated from human BCL contains a single long open reading frame encoding a 253-aa polypeptide. This sequence contains two potential translation initiation codons (residues 1 and 35) 5' to the N terminal sequence determined from the natural protein [64].

The upstream translation initiation codon is maintained in the p35 genes in non-human primates [65] and in pigs (Genbank accession number SSV08317), but not in mice [66] or several other mammals. The sequence initiated from the second methionine encodes a typical hydrophobic signal peptide (residues 35-56) with a consensus cleavage site immediately adjacent to the N-terminal sequence of the mature p35 protein. The hypothetical 34-aa sequence beginning with the methionine at residue 1 is less hydrophobic and includes several basic residues [64]. Based on the sequence data, together with the observation of the absence of the upstream initiation site in other species, and transfection data using cDNA lacking the methionine at residue 1, it was concluded that the second methionine is sufficient for expression of the functional p35 subunit [64]. However, other sequences similar to residues 1-34 in the IL-12 p35 are found linked to signal peptides of membrane-associated proteins, raising the possibility that this sequence may be involved in generating a membrane form of p35. The possible expression of IL-12 as a membrane-bound form in both human and murine macrophage cell lines has been suggested by an isolated study using cytofluorimetric staining with anti-IL-12 antibodies [67]. In addition, cloning of the feline IL-12 p35 chain revealed three leucine zipper motifs that are highly conserved in human and mouse [68], suggesting the potential for interactions between the p35 chain itself (homodimer), or between p35 and another leucine zipper-containing protein that may have biological activities yet ascribed to p35.

The promoter region of both the mouse and human p35 genes has been cloned [60-62]. We subcloned a 1143 bp BamHI-PstI fragment of the human gene. It contains some putative transcriptional motifs such as Sp1, IFN-γ-response element (γ-IRE), PU.1, C/EBP, GM-CSF, a T cell-specific transcription factor TCF-1α. The human p35 gene appears to initiate its transcription from at least two sites, one for B lymphoblastoid cells, and one for monocytes [62]. The latter follows a "TATA" box-like sequence which in the mouse gene has been suggested to be part of an ancestral IL-12 p35 promoter [61] and which would generate a shorter mRNA including only the second methionine at residue 35. The presence of multiple transcription initiation sites in the human and murine IL-12 p35 promoter raises an interesting prospect of different cell type usage of the promoter. The 1143bp p35 genomic fragment was linked to a luciferase reporter construct, transiently transfected into RPMI-8866 (EBV-B cell line that constitutively produce IL-12), Jurkat (T cell line that does not produce IL-12 but expresses the p35 gene constitutively), and RAW 264.7 cells which produce IL-12 only after stimulation with IFN-γ and LPS. This promoter construct is very active in B cells but not in T cells, suggesting that the T-cell specific *cis* elements may be absent in this promoter region. TPA does not further activate the promoter. It is also inducible in RAW 264.7 cells by a combination of IFN-γ and LPS treatment, mimicking that of the p40 gene.

DUAL REGULATION OF IL-12 PRODUCTION BY IL-4 AND IL-13.

The Th-2 cytokines IL-4 and IL-13 are generally grouped as anti-inflammatory or macrophage deactivating cytokines. However, studies from many laboratories including ours, clearly indicate that their actions are very complex and may result often in unexpected discordant effects. On monocytes/ macrophages, IL-4 inhibits the expression of IFN-γ-induced genes and the differentiation of these cells, whereas IFN-γ induces their differentiation. IL-4 or IL-13 treatment of monocytes induces the formation of long cytoplasmic protrusions that tightly adhere to substrates, giving them a dendritic-like appearance [69]. Addition of IL-4 to long-term monocyte cultures grown in the presence of GM-CSF blocks the differentiation to macrophages and the expression of macrophage markers such as CD14 or Fc receptors, while inducing cells that resemble dendritic/Langerhans cells with respect to antigenic phenotype (e.g. expression of CD1) and potent APC functions [70, 71]. Short-term treatment of monocytes with IL-4 or IL-13 enhances the expression of the surface antigens MHC class II, CD11b, CD11c, CD18, and CD23, but downregulates the expression of CD16, CD32, and CD64 [72-79]. Furthermore, IL-4 and IL-13 inhibit production of IL-1α and β, IL-6, IL-8, IL-10, IL-12 p35 and p40, GM-CSF, M-CSF, G-CSF, IFN-α and TNF-α, but enhance production of IL-1 receptor antagonist (IL-1Ra) by monocytes [78-83]. However, pretreatment of PBMC with IL-4 or IL-13 for 20 h or more enhances LPS- or SAC-stimulated production of IL-12 and TNF-α [84]. In mouse spleen cells, IL-4 added at the time of addition of the stimulus inhibited IL-12 production induced by the T cell-independent pathway (IFN-γ+LPS), but potently stimulated IL-12 production induced through the T cell-dependent pathway (CD40-CD40L) [85].

INHIBITION OF IL-12 PRODUCTION BY IL-10.

We have previously established that the inhibitory effects of IL-10 on IL-12 production from phagocytic cells are transcriptionally exerted and that this effect is an active process dependent on de novo protein synthesis [59]. However, a thorough examination of IL-12 p40 promoter activity in both

transient and stable transfection in RAW 264.7 cells treated with IFN-γ/LPS and IL-10 failed to localize an *IL-10*-responsive element along stretches of the human IL-12 p40 gene spanning from 11.3 kb upstream and some 5 kb downstream of the transcription initiation site (our unpublished data), suggesting that these regions of the IL-12 p40 promoter may not contain the IL-10-responsive elements.

INHIBITION OF IL-12 MEDIATED *VIA* FC RECEPTORS.

In collaboration with Dr. D. Mosser at Temple University School of Medicine, we examined further the initial observation that the ligation of immunocomplexes mimicked by IgG-opsonized erythrocytes with FcR resulted in severe and selective inhibition of IL-12 in bone marrow-derived mouse macrophages (BMM) [36]. It has become quite clear that this inhibition is transcriptional for both IL-12 p35 and p40 genes. An analysis of the nuclear binding activities to the p40 promoter indicated that NFkB (consisting of mainly p50, p65, and c-Rel), F1 and F2 binding activities were specifically inhibited by FcR ligation. This may be one of the mechanisms in which cross-linking of the FcR leads to selective downregulation of the *IL-12* p40 gene.

INHIBITION OF IL-12 PRODUCTION BY VIRUS-INDUCED IFN-α.

The role of IL-12 in viral infection is only beginning to be understood. IL-12 contributes primarily to the early development and activation of the innate immune response in a murine influenza virus infection model [86, 87], and during murine cytomegalovirus (MCMV) infection [88, 89]. In contrast, studies of IL-12 administration during lymphocytic choriomeningitis virus (LCMV) infections of mice have demonstrated that high concentrations of IL-12 may contribute to systemic toxicity and inhibition of the expansion of protective CTL [90, 91]. HIV infections are also associated with protective CTL [92-95], and cells from HIV-infected individuals have a deficiency in their ability to produce IL-12 [96, 97]. These observations suggest that during these infections, the levels of the endogenously produced IL-12 have to be tightly controlled. One of the conditions distinguishing viral infections from intracellular bacterial and parasitic infections is the early induction of systemic production of IFN-α/β [98-100]. Recently, Cousens et al [101] reported that exogenous IFN-α/β inhibited SAC-induced IL-12 production in murine splenic leukocytes, and this inhibition is specific for IL-12 since the induction of TNF-α was not affected and that of IL-6 was enhanced. They further observed that endogenous IFN-α/β induced by LCMV inhibited *in vitro* LPS-stimulated IL-12 production, which could be reversed by the addition of neutralizing antibodies against IFN-α/β. These results demonstrate a new pathway for regulating cytokine gene expression, IL-12 in particular, during anti-viral immune responses.

We have initiated some preliminary experiments to explore the possibility of examining the role of IFN-α in regulating IL-12 production in human monocytes. First, monocytes were pretreated with IFN-α for 16 h, followed by stimulation with LPS or SAC. No significant effect of IFN-α was seen on LPS- or SAC-stimulated IL-12 p40 and p70 production. However, when monocytes were pretreated with both IFN-α and IFN-γ together simultaneously, IFN-α inhibited LPS-, and SAC-induced IL-12 p40 and p70 enhanced by IFN-γ. This inhibition was also observed by sequential pretreatment with either IFN-γ first followed by IFN-α treatment, or IFN-α first, then IFN-γ treatment. These observations suggest that IFN-α pretreatment does not inhibit LPS- or SAC-induced IL-12 production but strongly antagonizes IFN-γ-potentiated IL-12 synthesis.

SUPPRESSION OF IL-2 PRODUCTION DURING ENDOTOXIN TOLERANCE.

Endotoxin tolerance is a clinically definable state in which exposure to low doses of bacterial toxins such as LPS leads to the secondary down-regulation of a variety of endotoxin-driven inflammatory responses such as fever, cachexia, shock, and lethality. Endotoxin tolerance may provide protection from the development of septic shock. However, a significant number of survivors of acute septic shock have persistent abnormalities of monocyte/macrophage activation, i.e. anergy and markedly elevated risks of delayed mortality from secondary infections. Their monocytes display dramatically reduced levels of proinflammatory cytokine production and HLA-DR expression along with a diminished capacity for antigen presentation [102-104]. This hypo-inflammatory state following sepsis has been described as "immunologic paralysis". It is notable that the phenotype of endotoxin tolerance, both *in vivo* and *in vitro*, is not simply that of a global deactivation of monocytic functions. The LPS-induced production of several proinflammatory (TNF-α, IL-1, IL-6) and anti-inflammatory (IL-10) cytokines is suppressed in endotoxin [105-107]. Production of these cytokines is also inhibited in response to heterologous inflammatory stimuli such as TSST-1 and SAC. However, the production of other mediators, including the anti-inflammatory cytokine receptor IL-1R antagonist, remains unaltered or enhanced [106, 108]. The mechanisms responsible for the generation of this characteristic functional state of monocytes/macrophages are beginning to be elucidated. Most work has focused on characterizing the altered regulation of TNF-α production during endotoxin tolerance. The down-regulation of the receptor for LPS binding and

signaling, CD14, does not appear to be a prerequisite for endotoxin tolerance [109, 110] and there is a CD14-independent mechanism for establishing LPS-tolerance, mediated through a protein kinase C (PKC)-dependent pathway [110]. Recent data indicate that IL-10, and secondarily, TGFβ, plays a central role in the TNF-α suppression seen during endotoxin tolerance in humans [106]. The observation that IL-10-deficient mice are not compromised in their ability to undergo endotoxin tolerance for TNF-α production [111] suggests that there are major species differences or redundant mechanisms in the regulation of endotoxin tolerance.

The production of IL-12 by monocytes/ macrophages and other antigen presenting cells is central to the generation of both innate and acquired cell-mediated immune responses to many pathogens. Both the proinflammatory and immunoregulatory roles of IL-12 are mediated through its stimulation of IFN-γ production from NK and T cells. IFN-γ and TNF-α have been strongly implicated as key mediators of endotoxic lethality[112, 113]. Therefore, the role of IL-12 in endotoxic shock and tolerance is of significant interest. IL-12 is produced early after *in vivo* exposure to LPS [114, 115]. In murine models, IL-12 plays a central role in the pathogenesis of the generalized Schwartzman reaction [116].

Furthermore, we have shown that IL-12 can prime for endotoxic shock by stimulating IFN-γ and TNF-α production [115], and neutralization of IL-12 protects mice from lethal endotoxemia [115, 117, 118]. Given the critical role of IL-12 in host responses to endotoxins and a wide range of other pathogens, we explored the regulation of IL-12 during endotoxin tolerance.

Primary human monocytes were preincubated with a low dose of LPS (1 ng/ml), which mimics blood endotoxin concentrations seen in severe sepsis [119], for 24 hours prior to secondary stimulation with a higher dose of LPS (1μg/ml) or SAC. IL-12 p40 was markedly inhibited by the treatment of LPS predosing. IFN-γ treatment during secondary stimulation completely reversed the inhibition of TNF-α production but had no significant effect on IL-12 p40, and especially on p70. Inclusion of neutralizing antibody to IL-10, but not to TGFβ, during the LPS-predosing period could, as has been shown previously [106], completely prevent LPS-induced suppression of TNF-α, and partially IL-12 p40 production. Under conditions of stimulation that lead to production of the functional IL-12 heterodimer (IFN-γ plus LPS or SAC), however, neutralization of IL-10 and TGFβ failed to fully prevent endotoxin tolerance-mediated suppression of IL-12 p40. Interestingly, under these same conditions production of the IL-12 heterodimer remained >95% inhibited, despite neutralization of both IL-10 and TGFβ, implying that IL-12 p35 subunit may be a limiting factor in this situation. Analysis of steady-state levels of human IL-12 p40 and p35 and TNF-α mRNA after LPS tolerance and SAC as the secondary stimulator indicated that SAC- and IFN-γ/SAC-induced IL-12 p40 and p35 mRNA appear to be completely suppressed during LPS tolerance whereas IFN-γ/SAC-induced TNF-α seems to be only partially inhibited. This result was mirrored by examining the transcription rate of these three genes in human monocytes in a nuclear run-on assay. Again, IL-12 p40 and p35 gene transcriptions are more significantly affected during LPS tolerance than that of TNF-α, which concurs with previous reports that IFN-γ treatment could reverse LPS tolerance-induced suppression of TNF-α production [104, 107, 120].

Production of proinflammatory cytokines by phagocytic cells during infectious or other pathological insults initiates the cascade of complex reactions leading to inflammation and to the activation of the effector cells of the innate resistance. The early inflammatory responses also set the stage for the generation of the antigen-specific effector cells of adaptive immunity, e.g. by directing the generation of Th1 or Th2 response, depending on the cytokine milieu at the response site. Our studies [121, 122] and those of others [123, 124] have revealed an important cross-talk between the effector cells of innate resistance/inflammation and those of adaptive immunity, with reciprocal regulation of both differentiation and activation, mediated by direct cellular interaction, cytokines, pharmacological mediators, and also by antibodies and complement components. IL-12 plays a major role in this cross-talk. The differentiation and activation of phagocytes to cells with differential ability to produce IL-12 and other proinflammatory/ regulatory cytokines is mediated during inflammation by cytokines as well as by interaction of cellular receptors with other reactive products, with a significant impact on the generation of innate and/or adaptive immune responses

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REFERENCES

1. Trinchieri G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol.* 13: 251.

2. Brunda M J, Luistro L, Warrier R R, Wright R B, Hubbard B R, Murphy M, Wolf S F, and Gately M K. 1993. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med.* 178: 1223.

3. Nastala C L, Edington H D, McKinney T G, Tahara H, Nalesnik M A, Brunda M J, Gately M K, Wolf S F, Schreiber R D, Storkus W J, and Lotze M T. 1994. Recombinant IL-12 administration induces tumor regression in association with IFN-γ production. *J Immunol.* 153: 1697.

4. Zou J, Yamamoto N, Fujii T, Takenaka H, Kobayashi M, Herrmann S H, Wolf S F, Fujiwara H, and Hamaoka T. 1995. Systemic administration of rIL-12 induces complete tumor regression and protective immunity: response is correlated with a striking reversal of suppressed interferon-γ production by anti-tumor T cells. *Int Immunol.* 7: 1135.

5. Mu J, Zou J, Yamamoto N, Tsutsui T, Tai X, Kobayashi M, Herrmann S, Fujiwara H, and Hamaoka T. 1995. Administration of rIL-12 prevents outgrowth of tumor cells metastasizing spontaneously to lung and lymph nodes. *Cancer Res.* 55: 4404.

6. Lieberman M, Sigal R, Williams N, Daly J. 1991. Natural killer cell stimulatory factor (NKSF) augments natural killer cell and antibody-dependent tumoricidal response against colon carcinoma cell lines. *J Surg Res* (USA). 50: 410.

7. Andrews J V, Schoof D D, Bertagnolli M M, Peoples G E, Goedegebuure P S, and Eberlein T J. 1993. Immunomodulatory effects of interleukin-12 on human tumor-infiltrating lymphocytes. *J Immunother.* 14: 1.

8. Bigda J, Mysliwska J, Dziadziuszko R, Bigda J, Mysliwski A, and Hellmann A. 1993. Interleukin-12 augments natural killer-cell mediated cytotoxicity in hairy cell leukemia. *Leuk Lymph.* 10: 121.

9. Rossi A R, Pericle F, Rashleigh S, Janiec J, and Djeu J Y. 1994. Lysis of neuroblastoma cell lines by human natural killer cells activated by interleukin-2 and interleukin-12. *Blood.* 83: 1323.

10. Kuge S, Watanabe K, Makino K, Tokuda Y, Mitomi T, Kawamura N, Habu S,V Nishimura T. 1995. Interleukin-12 augments the generation of autologous tumor-reactive CD8+ cytotoxic T lymphocytes from tumor-infiltrating lymphocytes. *Jap J Cancer Res.* 86: 135.

11. Rashleigh S P, Kusher D I, Endicott J N, Rossi A R, and Djeu J Y. 1996. Interleukins 2 and 12 activate natural killer cytolytic responses of peripheral blood mononuclear cells from patients with head and neck squamous cell carcinoma. *Arch Otolaryn - Head Neck Surg.* 122: 541.

12. DeCesare S L, Michelini-Norris B, Blanchard D K, Barton D P, Cavanagh D, Roberts W S, Fiorica J V, Hoffman M S, and Djeu J Y. 1995. Interleukin-12-mediated tumoricidal activity of patient lymphocytes in an autologous in vitro ovarian cancer assay system. *Gyn Oncol.* 57: 86.

13. Hanagiri T, Takenoyama M, Yoshimoto T, Hirashima C, Yoshino I, Nakanishi K, Nagashima A, Nomoto K, and Yasumoto K. 1996. Effects of interleukin-12 on in vitro culture with interleukin-2 of regional lymph node lymphocytes from lung cancer patients. *Cancer Immunol Immunother.* 43: 87.

14. Uharek L, Zeis M, Glass B, Steinmann J, Dreger P, Gassmann W, Schmitz N, and Muller-Ruchholtz W. 1996. High lytic activity against human leukemia cells after activation of allogeneic NK cells by IL-12 and IL-2. *Leukemia.* 10: 1758.

15. Steger G G, Gnant M F, Djavanmard M P, Mader R M, Jakesz R, Pierce W, DeKernion JB, Figlin R, and Belldegrun A. 1997. The in vitro effects of interleukin-12 upon tumor-infiltrating lymphocytes derived from renal cell carcinoma. *J Can Res Clin Oncol.* 123: 317.

16. Hill L L, Perussia B, McCue P A, and Korngold R. 1994. Effect of human natural killer cells on the metastatic growth of human melanoma xenografts in mice with severe combined immunodeficiency. *Cancer Res.* 54: 763.

17. Iwanuma Y, Chen FA, Egilmez N K, Takita H, and Bankert R B. 1997. Antitumor immune response of human peripheral blood lymphocytes coengrafted with tumor into severe combined immunodeficient mice. *Cancer Res.* 57: 2937.

18. Kobayashi M, Fitz L, Ryan M, Hewick R M, Clark S C, Chan S, Loudon R, Sherman F, Perussia B, and Trinchieri G. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med.* 170: 827.

19. Stern A S, Podlaski F J, Hulmes J D, Pan Y E, Quinn P M, Wolitzky A G, Familletti P C, Stremlo D L, Truitt T, Chizzonite R, and Gately M K. 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc Natl Acad Sci USA.* 87: 6808.

20. Trinchieri G. 1994. Interleukin 12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T helper cells type 1 and cytotoxic lymphocytes. *Blood.* 84: 4008.

21. D'Andrea A, Rengaraju M, Valiante N M, Chehimi J, Kubin M, Aste-Amezaga M, Chan S H, Kobayashi M, Young D, Nickbarg E, Chizzonite R, Wolf S F, and Trinchieri G. 1992. Production of natural killer cell stimulatory factor (NKSF/IL-12) by peripheral blood mononuclear cells. *J Exp Med.* 176: 1387.

22. Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen M, Silverman G J, Lotz M, Carson D A, and Raz E. 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science.* 273: 352.

23. Hodge-Dufour J, Noble P W, Horton M R, Bao C, Wysocka M, Burdick M D, Strieter R M, Trinchieri G, and PurÈ E. 1997. Induction of IL-12 and chemokines by hyaluronan requires adhesion-dependent priming of resident but not elicited macrophages. *J Immunol.* 159: 2492.

24. Jacobson N G, Szabo S J, Weber-Nordt R M, Zhong Z, Schreiber R D, Darnell J E, Jr, and Murphy K M. 1995. Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription Stat3 and Stat4. *J Exp Med.* 181: 1755.

25. Cella M, Scheidegger D, Plamer-Lehmann K, Lane P, Lanzavecchia A, and Alber G. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help *via* APC activation. *J Exp Med.* 184: 747.

26. Yoshida A, Koide Y, Uchijima M, and Yoshida T O. 1994. IFN-gamma induces IL-12 mRNA expression by a murine macrophage cell line, J774. *Biochem Biophys Res Commun.* 198: 857.

27. Kubin M, Chow J M, and Trinchieri G. 1994. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor-α, and IL-1β production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. *Blood.* 83: 1847.

28. D'Andrea A, Aste-Amezaga M, Valiante N M, Ma X, Kubin M, and Trinchieri G. 1993. Interleukin-10 inhibits human lymphocyte IFN-γ production by suppressing natural killer cell stimulatory factor/interleukin-12 synthesis in accessory cells. *J Exp Med.* 178: 1041.

29. Kubin M, Kamoun M, and Trinchieri G. 1994. Interleukin-12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J Exp Med.* 180: 211.

30. Murphy E E, Terres G, Macatonia S E, Hsieh C, Mattson J, Lanier L, Wysocka M, Trinchieri G, Murphy K, O'Garra A. 1994. B7 and IL-12 cooperate for proliferation and IFN-γ production by mouse T helper clones that are unresponsive to B7 costimulation. *J Exp Med.* 180:223.

31. D'Andrea A, Ma X, Aste-Amezaga M, Paganin C, and Trinchieri G. 1995. Stimulatory and inhibitory effects of IL-4 and IL-13 on production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and TNF–α production. *J Exp Med.* 181: 537.

32. Karp C L, Wysocka M, Wahl L M, Ahearn J M, Cuomo P J, Sherry B, Trinchieri G, and Griffin D E. 1996. Mechanism of suppression of cell-mediated immunity by measles virus. *Science.* 273: 228.

33. Stoiber H, Clivio A, and Dierich MP. 1997. Role of complement in HIV infection. *Annu Rev Immunol.* 15: 649.

34. Bajaj G, Rattan A, and Ahmad P. 1990. Circulating immune complexes in tuberculosis-an indicator of activity. *Ind J Pediatr.* 57: 203.

35. Vlock D R. 1990. Immune complexes and malignancy. *Immunol Series.* 53: 555.

36. Sutterwala F S, Noel G J, Clynes R, and Mosser D M. 1997. Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J Exp Med.* 185: 1977.

37. Marth T, and Kelsall B L. 1997. Regulation of interleukin-12 by complement receptor 3 signaling. *J Exp Med* 185: 1987

38. Berger S, Chandra R, BallÛ H, Hildenbrand R, and Stutte H J. 1997. Immune complexes are potent inhibitors of interleukin-12 secretion by human monocytes. *Eur J Immunol.* 27: 2994.

39. Gadd S J, Eher R, Majdic O, and Knapp W. 1994. Signal transduction via Fc gamma R and Mac-1 alpha-chain in monocytes and polymorphonuclear leucocytes. *Immunology* 81: 611.

40. Ohkuro M, Ogura-Masaki M, Kobayashi K, Sakai M, Takahashi K, and Nagasawa S. 1995. Effect of iC3b binding to immune complexes upon the phagocytic respose of human neutrophils: synergistic functions between Fc gamma R and CR3. *FEBS Letters.* 373: 189.

41. Zhou M J, and Brown E J. 1994. CR3 (Mac-1, alpha M beta 2, CD11b/CD18) and Fc gamma RIII cooperate in generation of a neutrophil respiratory burst: requirement for Fc gamma RIII and tyrosine phosphorylation. *J Cell Biol.* 125: 1407.

42. Stockl J, Majdic O, Pickl W F, Rosenkranz A, Prager E, Gschwantler E, and Knapp W. 1995. Granulocyte activation *via* a binding site near the C-terminal region of complement receptor type 3 alpha-chain (CD11b) potentially involved in intramembrane complex formation with glycosylphosphatidylinositol-anchored Fc gamma RIIIB (CD16) molecules. *J Immunol.* 154:5452.

43. Cassatella M A, Anegon I, Cuturi M C, Griskey P, Trinchieri G, and Perussia B. 1989. FcγR(CD16) interaction with ligand induces Ca2+ mobilization and phosphoinositide turnover in human natural killer cells. Role of Ca^{2+} in FcγR(CD16)-transcription and expression of lymphokine genes. *J Exp Med.* 169: 549.

44. Kuhnert P, Schalch L, and Jungi T W. 1990. Cytokine induction in human mononuclear cells stimulated by IgG-coated culture surfaces and by IgG for infusion. *Clin Immunol Immunopathol.* 57: 218.

45. Patry C, Herbelin A, Lehuen A, Bach J F, and Monteiro RC. 1995. Fc alpha receptors mediate release of tumour necrosis factor-alpha and interleukin-6 by human monocytes following receptor aggregation. *Immunology.* 86: 1.

46. Vossen A C, Tibbe G J, Kroos M J, van de Winkel J G, Benner R, and Savelkoul H F. 1995. Fc receptor binding of anti-CD3 monoclonal antibodies is not essential for immunosuppression, but triggers cytokine-related side effects. *Eur J Immunol.* 25:-1492.

47. Polat G L, Laufer J, Fabian I, Passwell J H. 1993. Cross-linking of monocyte plasma membrane Fc alpha, Fc gamma or mannose receptors induces TNF production. *Immunology.* 80: 287.

48. Balboa MA, Balsinde J, Aramburu J, Mollinedo F, and Lopez-Botet M. 1992. Phospholipase D activation in human natural killer cells through the Kp43 and CD16 surface antigens takes place by different mechanisms. Involvement of the phospholipase D pathway in tumor necrosis factor alpha synthesis. *J Exp Med.* 176: 9.

49. Simms H H, Gaither T A, Fries L F, and Frank M M. 1991. Monokines released during short-term Fc gamma receptor phagocytosis up-regulate polymorphonuclear leukocytes and monocyte-phagocytic function. *J Immunol.* 147: 265.

50. Stein M, and Gordon S. 1991. Regulation of tumor necrosis factor (TNF) release by murine peritoneal macrophages: role of cell stimulation and specific phagocytic plasma membrane receptors. *Eur J Immunol.* 21: 431.

51. Debets J M, van de Winkel J G, Ceuppens J L, Dieteren I E, and Buurman W A. 1990. Cross-linking of both Fc gamma RI and Fc gamma RII induces secretion of tumor necrosis factor by human monocytes, requiring high affinity Fc-Fc gamma R interactions. Functional activation of Fc gamma RII by treatment with proteases or neuraminidase. *J Immunol.* 144: 1304.

52. Daeron M. 1997. Fc receptor biology. *Annu Rev Immunol.* 15: 203.

53. Ma X, Chow J M, Gri G, Carra G, Gerosa F, Wolf S F, Dzialo R, and Trinchieri G. 1996. The interleukin-12 p40 gene promoter is primed by interferon-γ in monocytic cells. *J Exp Med.* 183: 147.

54. Murphy T L, Cleveland M G, Kulesza P, Magram J, and Murphy KM. 1995. Regulation of interleukin 12 p40 expression through an NF-κB half-site. *Mol Cell Biol.* 15: 5258.

55. Taki S, Sato T, Ogasawara K, Fukuda T, Sato M, Hida S, Suzuki G, Mitsuyama M, Shin E H, Kojima S, Taniguchi T, and Asano Y. 1997. Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. *Immunity.* 6: 673.

56. Holtschke T, Lohler J, Kanno Y, Fehr T, Giese N, Rosenbauer F, Lou J, Knobeloch K P, Gabriele L, Waring J F, Bachmann M F, Zinkernagel R M, Morse H C 3d, Ozato K, and Horak I. 1996. Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. *Cell.* 87: 307.

57. Scharton-Kersten T, Contursi C, Masumi A, Sher A, and Ozato K. 1998. ICSBP-deficient mice display impaired resistance to intracellular infection due to a primary defect in IL-12 p40 induction. *J Exp Med.* in press.

58. Wang I, Ma X, Contursi C, Masumi A, Lo J, Trinchieri G, and Ozato K. 1997. A critical role of interferon consensus sequence binding protein (ICSBP) in the activation of IL-12 p40 gene promoter. *J Interf Cykokine Res.* 17: 576.

59. Aste-Amezaga M, Ma X, Sartori A, and Trinchieri G. 1998. Molecular mechanisms of the induction of interleukin-12 and its inhibition by IL-10. *J Immunol.* in press.

60. Tone Y, Thompson SA, Babik J M, Nolan K F, Tone M, Raven C, and Waldmann H. 1996. Structure and chromosomal location of the mouse interleukin-12 p35 and p40 subunit genes. *Eur J Immunol.* 26: 1222.

61. Yoshimoto T, Kojima K, Funakoshi T, Endo Y, Fujita T, and Nariuchi H. 1996. Molecular cloning and characterization of murine IL-12 genes. *J Immunol.* 156: 1082.

62. Hayes M P, Murphy F J, and Burd P R. 1998. Interferon-gamma-dependent inducible expression of the human interleukin-12 p35 gene in monocytes initiates from a TATA-containing promoter distinct from the CpG-rich promoter active in EBV-transformed lymphoblastoid cells. *Blood.* in press.

63. Schoenhaut D S, Chua A O, Wolitzky A G, Quinn P M, Dwyer C M, McComas W, Familletti P C, Gately M K, and Gubler U. 1992. Cloning and expression of murine IL-12. *J Immunol.* 148: 3433.

64. Wolf S F, Temple P A, Kobayashi M, Young D, Dicig M, Lowe L, Dzialo R, Fitz L, Ferenz C, Hewick R M, Kelleher K, Herrmann S H, Clark S C, Azzoni L, Chan S H, Trinchieri G, and Perussia B. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J Immunol.* 146: 3074.

65. Villinger F, Brar S S, Mayne A, Chikkala N, and Ansari AA. 1995. Comparative sequence analysis of cytokine genes from human and nonhuman primates. *J Immunol.* 155: 3946.

66. Gubler U, Chua A O, Schoenhaut D S, Dwyer C M, McComas W, Motyka R, Nabavi N, Wolitzky A G, Quinn P M, Familletti P C, and Gately M K. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci USA.* 88: 4143.

67. Fan X, Sibalic V, Niederer E, and Wuthrich R P. 1996. The proinflammatory cytokine interleukin-12 occurs as a cell membrane-bound form on macrophages. *Biochem Biophys Res Commun.* 225: 1063.

68. Bush K, Day N K, Kraus L A, Good R A, and Bradley W G. 1994. Molecular cloning of feline interleukin 12 p35 reveals the conservation of leucine-zipper motifs present in human and murine IL-12 p35. *Mol Immunol.* 31: 1373.

69. de Waal Malefyt R, Figdor C G, and de Vries J E. 1993. Effects of interleukin 4 on monocyte functions: comparison to interleukin 13. *Res Immunol.* 144: 629.

70. Sallusto F, and Lanzavecchia A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macro-phage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med.* 179: 1109.

71. Peters J H, Ruppert J, Giesler R K, Najar H M, and Xu H. 1991. Differentiation of human monocytes into CD14 negative accessory cells: do dendritic cells derive from the monocytic lineage? *Pathobiology.* 59: 122.

72. Vercelli D, Jabara H H, Lee B, Woodland N, Geha RS, and Leung DYM. 1988. Human recombinant IL-4 induces FceR2/CD23 on normal human monocytes. *J Exp Med.* 167: 1406.

73. Te Velde A A, Rousset F, Peronne C, de Vries J E, and Figdor C G. 1990. IFN-α and IFN-γ have different regulatory effects on IL-4 induced membrane expression of FceRIIβ and release of soluble FceRIIb by human monocytes. *J Immunol.* 144: 3052.

74. Te Velde A A, Klomp J P, Yard B A, de Vries J E, and Figdor C G. 1988. Modulation of phenotypic and

functional properties of human peripheral blood monocytes by IL-4. *J Immunol.* 140: 1548.

75. Becker S, and Daniel E G. 1990. Antagonistic and additive effects of IL-4 and IFNγ on human monocytes and macrophages: Effects on Fc receptors, HLA-D antigens, and superoxide production. *Cell Immunol.* 129: 351

76. Littman B H, Dastvan F F, Carlson P L, and Sanders K M. 1989. Regulation of monocyte/ macrophage C2 production and HLA-DR expression by IL-4 (BSF-1) and IFNγ. *J Immunol.* 142:520.

77. Gerrard T L, Dyer D R, and Mostowski H S. 1990. IL-4 and granulocyte-macrophage colony stimulating factor selectively increase HLA-DR and HLA-DP antigens, but not HLA-DQ antigens on human monocytes. *J Immunol.* 144: 4670.

78. Bogdan C, and Nathan C. 1993. Modulation of macrophage function by transforming growth factor β, interleukin-4, and interleukin-10. *Ann NY Acad Sci.* 685:7 13.

79. de Waal Malefyt R, Figdor C-G, Huijbens R, Mohan-Peterson S, Bennett B, Culpepper J, Dang W, Zurawski G, de Vries J-E. 1993. Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. *J Immunol.* 151: 6370.

80. Donnelly R P, Fenton M J, Kaufman J D, and Gerrard TL. 1991. IL-1 expression in human monocytes is transcriptionally and posttranscriptionally regulated by IL-4. *J Immunol.* 146: 3431

81. Hart P H, Vitti G F, Burgess D R, Whitty G A, Piccoli D S, and Hamilton J A. 1989. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor a, interleukin 1 and prostaglandin E2. *Proc Natl Acad Sci USA.* 86: 3803.

82. Essner R, Rhoades K, McBride WH, Morton DL, and Economou JS. 1989. IL-4 down-regulates IL-1 and TNF gene expression in human monocytes. *J Immunol.* 142: 3857.

83. Te Velde A A, Huijbens R J, de Vries J E, and Figdor C G. 1990. Interleukin 4 inhibits secretion of IL-1β, tumor necrosis factor α, and IL-6 by human monocytes. *Blood.* 76: 1392.

84. Marshall J D, Robertson S E, Trinchieri G, and Chehimi J. 1997. Priming with IL-4 and IL-13 during HIV-1 infection restores *in vitro* IL-12 production by mononuclear cells of HIV-infected patients. *J Immunol.* 159: 5705.

85. Thomas H, Hanby A M, Smith R A, Hagger P, Patel K, Raikundalia B, Camplejohn R S, and Balkwill F R. 1996. An inbred colony of oncogene transgenic mice: diversity of tumours and potential as a therapeutic model. *Br J Cancer.* 73: 65.

86. Monteiro J M, and Trinchieri G. 1996. Does IL-12 play a role in the viral immune response? *Ann NY Acad Sci.* 795: 366.

87. Monteiro J M, Harvey C, and Trinchieri G. 1998. The role of interleukin-12 in primary influenza virus infection. *J Virol.* in press.

88. Orange J S, and Biron C A. 1996. Characterization of early IL-12, IFN-alpha/beta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *J Immunol.* 156:4746.

89. Orange J S, and Biron C A. 1996. An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *J Immunol.* 156: 1138.

90. Orange J S, Wolf S F, and Biron C A. 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. *J Immunol.* 152: 1253.

91. Orange J S, Salazar-Mather T P, Opal S M, Spencer R L, Miller A H, McEwen B S, and Biron CA. 1995. Mechanism of interleukin 12-mediated toxicities during experimental viral infections: role of tumor necrosis factor and glucocorticoids. *J Exp Med.* 181: 901.

92. Kalams S A, and Walker B D. 1994. The cytotoxic T-lymphocyte response in HIV-1 infection. *Clin Lab Med.* 14: 271.

93. Levy JA. 1993. HIV pathogenesis and long-term survival. *AIDS.* 7: 1401.

94. Ennen J, Findeklee H, Dittmar M T, Norley S, Ernst M, and Kurth R. 1994. CD8+ T lymphocytes of African green monkeys secrete an immunodeficiency virus suppressing lymphokine. *Proc Natl Acad Sci USA.* 91: 7207.

95. Baier M, Werner A, Bannert N, Metzner K, and Kurth R. 1995. HIV suppression by interleukin-16. *Nature.* 378: 563.

96. Chehimi J, Starr S, Frank I, D'Andrea A, Ma X, MacGregor RR, Sennelier J, and Trinchieri G. 1994. Impaired interleukin-12 production in human immunodeficiency virus-infected patients. *J Exp Med.* 179: 1361.

97. Chehimi J, and Trinchieri G. 1994. Interleukin-12: a bridge between innate resistance and adaptive immunity with a role in infection and acquired immunodeficiency. *J Clin Immunol.* 14: 149.

98. Welsh R M Jr. 1978. Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. I. Characterization of natural killer cell induction. *J Exp Med.* 148: 163.

99. Grundy J E, Trapman J, Allan J E, Shellam G R, and Melief C J. 1982. Evidence for a protective role of interferon in resistance to murine cytomegalovirus and its control by non-H-2-linked genes. *Infect Immun.* 37: 143.

100. Muller U, Steinhoff U, Reis L F, Hemmi S, Pavlovic J, Zinkernagel R M, and Aguet M. 1994. Functional role of type I and type II interferons in antiviral defense. *Science.* 264: 1918.

101. Cousens L P, Orange J S, Su H C, and Biron C A. 1997. Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production *in vitro* and endogenously during viral infection. *Proc Natl Acad Sci USA*. 94: 634.

102. Volk H D, Thieme M, Ruppe U, Heym S, D"cke W, Manger D, Zuckerman S, Golosubow A, Nieter B, and Klug H. 1993. Alterations in function and phenotype of monocytes from patients with septic disease: predictive value and new therapeutic strategies. In Host Defense Dysfunction in Trauma, Shock, and Sepsis Faist E, Meakins J L, Eds, Berlin, Germany: Springer-Verlag. 246.

103. Docke W D, Syrbe U, Meinecke A, Platzer C, Makki A, Asadullah K A, Klug C, Zuckerman H, Reinke P, and Brunner H. 1994. Improvement of monocytic function: a new therapeutic approach? In Sepsis: Current Perspectives in Pathophysiology and Therapy Reinhart K, Eyrich K, Sprung C, Eds, Berlin, Germany: Springer-Verlag. 437.

104. Docke W D, Randow F, Syrbe U, Krausch D, Asadullah K A, Reinke P, Volk H, Kox W. 1997. Monocyte deactivation in septic patients: restoration by IFN-γ treatment. *Nature Medicine*. 3: 678.

105. Ziegler-Heitbrock H W, Blumenstein M, Kafferlein E, Kieper D, Petersmann I, Endres S, Flegel W A, Northoff H, Riethmuller G, and Haas JG. 1992. *In vitro* desensitization to lipopolysaccharide suppresses tumour necrosis factor, interleukin-1 and interleukin-6 gene expression in a similar fashion. *Immunology*. 75: 264.

106. Randow F, Syrbe U, Meisel C, Krausch D, Zuckermann H, Platzer C, and Volk H D. 1995. Mechanisms of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor β. *J Exp Med*. 181: 1887.

107. Randow F, Docke W D, Bundschuh D S, Hartung T, Wendel A, Volk H D. 1997. *In vitro* prevention and reversal of lipopolysaccharide desensitization by IFN-gamma, IL-12, and granulocyte macrophage colony-stimulating factor. *J Immunol*. 158: 2911.

108. Poll T V, Coyle S M, Moldawer L L, Lowry S F. 1996. Changes in endotoxin-induced cytokine production by whole blood after *in vivo* exposure of normal humans to endotoxin. *J Inf Dis*. 174: 1356.

109. Fahmi H, and Chaby R. 1993. Desensitization of macrophages to endotoxin effects is not correlated with a down-regulation of lipopolysaccharide binding sites. *Cell Immunol*. 150: 219.

110. Labeta M O, Durieux J J, Spagnoli G, Fernandez N, Wijdenes J, and Herrmann R. 1993. CD14 and tolerance to lipopolysaccharide: biochemical and functional analysis. *Immunology*. 80: 415.

111. Berg D J, Kuhn K, Rajewsky W, Muller S, Menon N, Davidson G, Grunig G, and Rennick D. 1995. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and Schwartzman reaction but not endotoxin tolerance. *J Clin Invest*. 96: 2339.

112. Dinarello C A. 1991. The proinflammatory cytokines interleukin-1 and tumor necrosis factor and treatment of the septic shock syndrome. *J Inf Dis*. 163: 1177.

113. Heinzel F P. 1990. The role of IFN-γ in the pathology of experimental endotoxemia. *J Immunol* 145: 2920

114. Heinzel F P, Rerko R M, Ling P, Hakimi J, and Schoenhaut D S. 1994. Interleukin 12 is produced *in vivo* during endotoxemia and stimulates synthesis of gamma interferon. *Infect Immun*. 62: 4244.

115. Wysocka M, Kubin M, Vieira L Q, Ozmen L, Garotta G, Scott P, and Trinchieri G. 1995. Interleukin-12 is required for interferon-g production and lethality in lipopolysaccharide-induced shock in mice. *Eur J Immunol*. 25: 672.

116. Ozmen L, Pericin M, Hakimi J, Chizzonite R A, Wysocka M, Trinchieri G, Gately M, and Garotta G. 1994. IL-12, IFN-γ and TNF-α are the key cytokines of the generalized Shwartzman reaction. *J Exp Med*. 180: 907.

117. Mattner F, Ozmen L, Podlaski F J, Wilkinson V L, Presky D H, Gately M K, and Alber G. 1997. Treatment with homodimeric interleukin-12 (IL-12) p40 protects mice from IL-12-dependent shock but not from tumor necrosis factor alpha-dependent shock. *Infect Immun*. 65: 4734.

118. Zisman D A, Kunkel S L, Strieter R M, Gauldie J, Tsai W C, Bramson J, Wilkowski J M, Bucknell K A, and Standiford T J. 1997. Anti-interleukin-12 therapy protects mice in lethal endotoxemia but impairs bacterial clearance in murine Escherichia coli peritoneal sepsis. *Shock*. 8: 349.

119. Brandzaeg P, Kierulf P, Gaustad P, Skulberg A, Bruun J N, Halvorsen S, and Sorensen E. 1989. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. *J Infect Dis*. 159: 195.

120. Haas J G, Baeuerle P A, Riethmuller G, and Ziegler-Heitbrock H W. 1990. Molecular mechanisms in down-regulation of tumor necrosis factor expression. *Proc Natl Acad Sci USA*. 87: 9563.

121. Trinchieri G, Murphy M, and Perussia B. 1987. Regulation of hematopoiesis by T lymphocytes and natural killer cells. *CRC Critical Reviews in Oncology/Hematology*. 7: 219.

122. Trinchieri G, Kubin M, Bellone G, and Cassatella M C. 1993. Cytokine cross-talk between phagocytic cells and lymphocytes: relevance for differentiation/ activation of phagocytic cells and regulation of adaptive immunity. *J Cell Biochem*. 53: 301.

123. Tripp CS, and Unanue ER. 1995. Macrophage production of IL-12 is a critical link between the innate and specific immune responses to *Listeria*. *Res Immunol*. 146:515.

124. Hunter C A, Bermudez L, Beernink H, Waegell W, and Remington J S. 1995. Transforming growth factor-beta inhibits interleukin-12-induced production of interferon-gamma by natural killer cells: a role for transforming growth factor-beta in the regulation of T cell-independent resistance to *Toxoplasma gondii*. *Eur J Immunol*. 25: 994

The taming of IL-12: suppressing the production of proinflammatory cytokines

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Abstract: Interleukin (IL)-12 is a cytokine that possesses both proinflammatory and immunoregulatory activity. IL-12, and the interferon-γ (IFN-γ) that is induced by IL-12, play central roles in the development of the Th1-type immune responses that are required for immunity to intracellular pathogens. Recently a number of these pathogens, including Leishmania, measles virus, and human immunodeficiency virus (HIV), have been shown to subvert the development of cell-mediated immunity by actively inhibiting the production of IL-12. Similarly, the ligation of phagocytic receptors on macrophages has also been shown to suppress IL-12 production. The suppression of IL-12 production by receptor ligation occurs by at least two distinct mechanisms: one involves a direct inhibition of gene transcription and the other depends on the production of inhibitory cytokines. We review studies in which IL-12 has been experimentally manipulated, and we compare the mechanisms by which this regulation can occur. Because the IL-12 that is produced during acute inflammation and chronic autoimmune disorders can lead to exacerbated disease, the development of pharmacological means to suppress IL-12 production is currently under investigation. This review focuses on the production of IL-12 by antigen-presenting cells and the methods by which the down-regulation of IL-12 production can be exploited either by pathogens or for therapeutic ends. *J. Leukoc. Biol.* 65: 543–551; 1999.

Key Words: *phagocytes · Th1 · lipopolysaccharide · macrophage · receptors*

PRODUCTION AND BIOLOGICAL FUNCTIONS OF INTERLEUKIN-12 (IL-12)

Biologically active IL-12 is a 70-kDa heterodimer (p70) composed of two covalently linked subunits, p35 and p40 [1, 2]. IL-12 was originally discovered as a product of B cell lines [3]. However, the most important producers of IL-12 appear to be monocytes, macrophages, and dendritic cells [4, 5]. Polymorphonuclear leukocytes have also been shown to produce IL-12 [6]. IL-12 production can be induced by exposing responsive cells to a variety of microbial products. Lipopolysaccharide (LPS) of gram-negative bacteria has been the most clearly defined inducer of IL-12 [4]. Both LPS and lipoteichoic acid from gram-positive bacteria have been shown to induce IL-12 primarily through their interaction with CD14 [7]. Trehalose dimycolate, a glycolipid present in the cell wall of *Mycobacterium tuberculosis* [8], and glycoprotein fractions from *Trypanosoma cruzi* trypomastigotes [9] and *Toxoplasma gondii* tachyzoites [10] have been shown to induce IL-12. Other inducers of IL-12 include the recombinant *Leishmania braziliensis* antigen LeIF [11] and the CpG motifs contained in bacterial DNA [12]. In addition to infectious agents, phagocyte interactions with inflammatory matrix molecules and with activated T cells are important inducers of IL-12. The interaction of macrophage CD44 with low-molecular-weight (inflammatory) fragments of the extracellular matrix glycosaminoglycan hyaluronan is capable of inducing IL-12 production [13]. Activated T cells have been shown to induce the production of IL-12 in monocytes by the interaction of T cell-associated CD40 ligand with CD40 on monocytes [14]. CD40-CD40 ligand interaction has also been shown to be an important inducer of IL-12 by dendritic cells [15, 16]. Thus, the direct interaction of phagocytic cells with microbial products or phagocyte interactions either with extracellular matrix components or with activated T cells can drive the initiation and the propagation of IL-12 production.

The role of IL-12 in inflammation and acquired immune responses has been well documented [17]. IL-12 drives the development of Th1-type immune responses [18–20] and is a potent inducer of interferon-γ (IFN-γ) from T cells and natural killer (NK) cells [1, 3, 21]. Both *in vitro* and *in vivo* experiments have demonstrated that IL-12 plays a crucial role in the development of specific immunity against a number of intracellular pathogens, including *Leishmania major* [22, 23], *Mycobacterium tuberculosis* [24], *Listeria monocytogenes* [18], and *Toxoplasma gondii* [25]. Animals lacking the IL-12 p40 gene [26] or animals treated with antibodies to IL-12 [23, 27, 28] are invariably more susceptible to infections with these intracellular pathogens. IL-12 has also been shown to have adjuvant properties, stimulating an effective cellular immune response to microbial antigens, which are not appropriately immunogenic

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Abbreviations: LPS, lipopolysaccharide; NK, natural killer; TNF-α, tumor necrosis factor α; PBMCs, peripheral blood mononuclear cells; IL, interleukin; IFN-γ, interferon-γ; IVIG, intravenous immune globulin; PGE_2, prostaglandin E_2.

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when administered alone [29]. The over-production of IL-12, however, has the potential to be detrimental to the host. IL-12 produced during LPS endotoxemia [30] and during a number of autoimmune disorders, including insulin-dependent diabetes mellitus [31], experimental allergic encephalomyelitis [32], or collagen-induced arthritis [33], can lead to exacerbated disease.

The production of IL-12 p40 is regulated primarily at the transcriptional level. The IL-12 p40 promoter contains multiple potential transcription-factor binding sites, three of which have been directly implicated in p40 gene regulation. An Ets-like element between -222 and -204 from the IL-12 p40 transcription start site has been identified by Ma et al. to be important for IL-12 p40 promoter activity [34]. This element interacts with a multi-subunit transcription complex termed F1. This complex is highly inducible by IFN-γ priming and LPS stimulation. The F1 complex is composed of Ets-2, IRF-1, c-Rel, and Ets-related factors [34]. An NF-κB half-site located at -117 to -107 in the p40 promoter has been identified by Murphy et al. [35] as being involved in responsiveness to LPS stimulation. In macrophage-like cell lines, both the NF-κB and Ets sites appear to be essential for the induction of p40 promoter activity because mutations of either the NF-κB or Ets site abolished p40 promoter activity in response to IFN-γ and LPS [36]. Recently, Plevy et al. have identified a site located between -96 and -88 of the murine p40 promoter, also conserved in the human p40 promoter, that can bind to members of the C/EBP family of transcription factors in activated macrophages [37]. Although no direct interactions between the C/EBP and Rel proteins were observed, the C/EBP element exhibits functional synergy with the upstream NF-κB half-site [37].

IL-12 secretion is also regulated at the level of p35 mRNA production. Because transcripts for the IL-12 p35 gene have been detected in a variety of cell types that do not secrete intact p70 heterodimer, p35 was originally thought to play only a minor role in regulating IL-12 biosynthesis. In monocytes, however, it is now clear that p35 transcription is tightly regulated [38–40]. Exposing phagocytic cells to IFN-γ causes an increase in p35 transcription after stimulation. In fact IFN-γ, which has only a priming effect on the p40 gene, can induce p35 gene transcription and mRNA accumulation in the absence of further stimulation [6]. The p35 promoter contains several putative elements that may contribute to regulation, including GAS, ICSBP, and ISRE elements [41, 42] in addition to multiple potential NF-κB sites. Unlike p40, which can be produced in large excess and secreted as monomers, as homodimers, or as the p70 heterodimer, the p35 subunit appears to be secreted only as part of the p70 heterodimer. Therefore, the production of p35 may be the rate-limiting step in controlling the production of biologically relevant IL-12 p70 by monocytes and macrophages [39]. Recent studies have identified a unique transcriptional start site of IL-12 p35 transcripts in monocytes relative to that observed in lymphoblastoid cells [40], resulting in a different translational product in these cells. Thus, the regulation of biologically active IL-12 p70 may occur not only at the transcriptional levels of both genes, but also post-transcriptionally due to variable associations of the different p35 products with the p40 chain [39].

Despite a dramatic increase in the transcription of the IL-12 p40 gene, resting cells make only low levels of biologically active IL-12 p70 after stimulation with microbial products. Cells primed with IFN-γ, however, secrete high levels of p70 in response to microbial products [38, 43]. Recently, Munder and colleagues [44] demonstrated that murine macrophages are themselves capable of producing IFN-γ after stimulation with a combination of IL-12 and IL-18. Thus, macrophage production of IFN-γ has the potential to provide an autocrine mechanism of macrophage activation, resulting in additional IL-12 p70 production.

SUPPRESSION OF IL-12 BY INTRACELLULAR PATHOGENS

The down-modulation of IL-12 production may be exploited by intracellular pathogens of macrophages as a way to escape cell-mediated immunity. To date, three intracellular pathogens, one parasite and two viruses, have been shown to suppress macrophage IL-12 production. The interaction of *Leishmania* spp. [45–47], measles virus [48], and HIV [49, 50] with macrophages and monocytes results in a marked decrease in IL-12 production. In all three cases, it has been postulated that this down-regulation of IL-12 may interfere with or delay the development of cell-mediated immunity to these pathogens.

Leishmania spp. are intracellular pathogens that reside primarily if not exclusively within host tissue macrophages. Activation of macrophages by IFN-γ is required to control the growth of this intracellular protozoan. IL-12-driven IFN-γ has been shown to play a crucial role in the resolution of a leishmanial infection. In a murine model of leishmaniasis, susceptible BALB/c mice treated with rIL-12 were rendered resistant to *L. major* due to an up-regulation of IFN-γ production and the initiation of a protective Th1-type response [22, 23]. Animals lacking the IL-12 p40 gene [26] or animals treated with antibodies to IL-12 are more susceptible to infection with *L. major* [27]. These studies demonstrate the importance of IL-12 to host defense in Leishmania infections. Despite this apparent importance, *L. major* fails to elicit IL-12 biosynthesis from purified macrophage monolayers *in vitro* [51]. Recent studies have taken these observations further and have shown that the phagocytosis of Leishmania promastigotes is associated with an active suppression of IL-12 production by macrophages [45]. In these studies, the addition of Leishmania to macrophages prevented IL-12 production when macrophages were stimulated with LPS or other stimuli. The *L. major* promastigotes used in these experiments were opsonized with complement and hence utilized complement receptor type 3 (CR3) as their principal mode of entry into macrophages [52]. We have compared the ability of opsonized and unopsonized parasites to inhibit macrophage IL-12 production in response to LPS *in vitro*. Unopsonized parasites bound poorly to macrophages and diminished LPS-driven IL-12 production only marginally, whereas parasites opsonized with complement bound avidly to macrophages and decreased IL-12 production to a greater extent (**Fig. 1**), confirming the original observation [45]. Recent studies by the same group have shown that macrophages taken from Leishmania lesions produced little

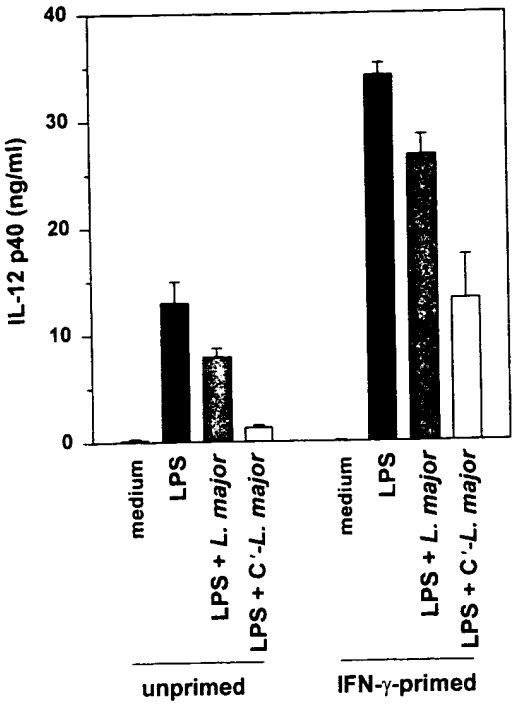

Fig. 1. Suppression of macrophage IL-12 p40 induction by *L. major* promastigotes. Bone marrow-derived macrophages from C57BL/6 mice that were either primed with IFN-γ (100 U/mL) for 6 h or left unprimed were stimulated with LPS (100 ng/mL) alone or LPS in combination with either unopsonized- (*L. major*) or complement opsonized- (C'-*L. major*) *L. major* promastigotes at an 8:1 ratio. Unstimulated cells were cultured in parallel without LPS or promastigotes. Twenty-four hours after stimulus addition the supernatant was harvested and IL-12 p40 levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the mean ± SD.

detectable IL-12 if they harbored parasite antigen [46]. Taken together, these observations suggest that the adhesion of *L. major* promastigotes or their entry into macrophages was required for the down-regulation of IL-12 production. Weinheber et al. have recently demonstrated that the amastigote form of both *L. mexicana* and *L. major* are also capable of suppressing macrophage IL-12 production induced by either LPS or CD40 receptor ligation [47]. The finding that *Leishmania* spp. can selectively suppress IL-12 production by macrophages may explain the delayed induction of IL-12 that is observed *in vivo* during leishmanial infections.

Infection with measles virus and immunization with live-attenuated measles vaccines have both been associated with a transient immunosuppression, characterized by abnormalities in cell-mediated immunity, diminished T cell proliferation in response to antigen, and the production of Th2-type cytokines [53]. Recent studies have shown that *in vitro* infection of human monocytes with measles virus can cause a marked defect in the ability of infected cells to produce IL-12 p40 and p70 [48]. There was some specificity to this inhibition because measles virus infection had little effect on the production of other proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and IL-6. The inhibition of IL-12 was correlated with the interaction of measles virus with the monocyte receptor CD46 (membrane cofactor protein). Furthermore, agonistic antibodies to CD46 or ligands of CD46 such as C3 fragments, were also capable of mediating the suppression of IL-12. The suppression in IL-12 induction by measles virus provides a possible mechanism by which measles virus can induce the immunosuppression that has been associated with those infections.

Cell-mediated immune responses play an important role in impeding the progression of HIV infection. During the course of HIV disease there is a progressive loss of Th1-type immune responses [54]. Peripheral blood mononuclear cells (PBMC) from HIV-positive patients produce 10-fold less IL-12 p40 and 5-fold less p70 heterodimer than did PBMC from healthy HIV-negative donors in response to *Staphylococcus aureus in vitro* [49]. The production of other proinflammatory cytokines, including TNF-α and IL-1β, were not affected by HIV infection, and the production of IL-6 was actually enhanced [49]. When human monocyte-derived macrophages were infected *in vitro* with the monocytotropic strain HIV_{Ba-L} and stimulated with *S. aureus* 2 weeks after the initial HIV_{Ba-L} infection, there was an inhibition of both p40 and p35 mRNA accumulation that correlated with a 12-fold decrease in IL-12 p70 production [50]. The possibility of IL-10 hypersecretion by HIV-infected PBMC as a mechanism for suppression of IL-12 remains unclear. Some studies have reported increased IL-10 production by PBMC from HIV-positive patients [50], whereas others have reported no change in IL-10 production [49]. The mechanism by which HIV suppresses IL-12 production is not clearly understood. HIV can efficiently activate complement and can utilize complement receptors to enhance infectivity [55], and it has been postulated that HIV may utilize complement opsonization as a way to suppress cell-mediated immunity [48]. The diminished production of IL-12 by PBMC from HIV-positive patients may have important implications for the progression of the HIV infection. In addition, the suppression of IL-12 production may contribute to the immunosuppression associated with HIV infections and lead to increased susceptibility to the opportunistic infections seen in AIDS. Gazzinelli and colleagues have shown that HIV infection can specifically suppress the production of IL-12 that normal macrophages make after exposure to *T. gondii* [56].

In summary, a number of intracellular pathogens have been shown to actively suppress the production of IL-12. In all three of the cases described to date, the inhibition was specific to IL-12 and not a global defect in cytokine production. However, clear distinctions between the three experimental systems suggest the possibility of multiple mechanisms to account for this suppression. In the Leishmania studies, parasites and stimuli (LPS) were added to macrophages simultaneously. Measles virus was added 2 days before stimuli, and in the HIV studies the virus was added 2 weeks before stimuli. A comprehensive examination of the infection conditions that result in IL-12 suppression has not been undertaken.

SUPPRESSION OF IL-12 THROUGH RECEPTOR LIGATION

The ligation of phagocytic receptors on human monocytes or murine macrophages has recently been shown to suppress IL-12 production. CR3 (CD11b/CD18, Mac-1) and the Fcγ receptors are the two phagocytic receptor classes that have

been shown to have the clearest role in down-regulating IL-12 in these systems [57, 58]. The ligation of either CR3 or FcγR with monoclonal antibodies or with particulate ligands results in a profound inability of monocytes or macrophages to produce IL-12 in response to a variety of proinflammatory stimuli, including LPS, *S. aureus*, and CD40 ligand [57, 58]. The suppression of IL-12 occurred even when ligands and stimuli were simultaneously added to macrophages, eliminating LPS tolerance as a possible explanation for this effect. This suppression was specific to IL-12 because the induction of other proinflammatory cytokines such as TNF-α and IL-6 were not affected. The receptor-mediated suppression of IL-12 was a direct inhibition of IL-12 transcription, and not dependent on novel protein synthesis or particle internalization [57]. Both the p40 and p35 genes were affected by receptor ligation (unpublished observations). The down-modulation of IL-12 after receptor ligation was independent of IL-10 production because the ligation of FcγR on macrophages from IL-10 gene knockout mice resulted in a suppression of IL-12 p40 mRNA that was similar in magnitude to that which occurs in wild-type macrophages (**Fig. 2**). A similar suppression of IL-12 has been observed after the ligation of CD46 on monocytes with agonistic antibodies to CD46 or ligands of CD46 such as C3 fragments [48].

The mechanisms for the suppression of IL-12 induction by receptor ligation are currently being studied; however, no consensus has yet emerged. Candidate mechanisms include alterations in protein phosphorylation, increases in intracellular cAMP, and alterations in calcium fluxes. The ligation of macrophage CR3 has been correlated with an inhibition of

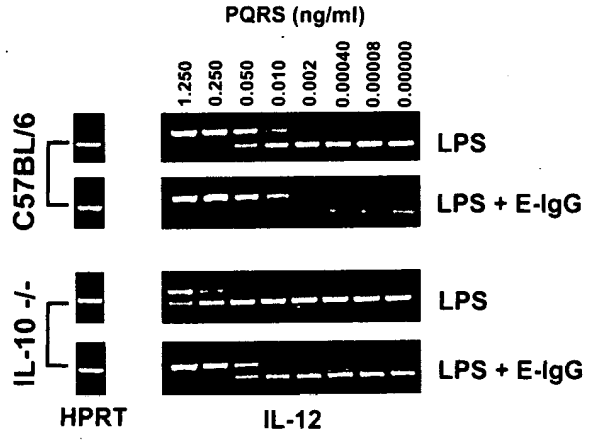

Fig. 2. FcγR ligation can suppress IL-12 induction independently of IL-10. Bone marrow-derived macrophages from C57BL/6 or IL-10$^{-/-}$ mice were exposed to either LPS (100 μg/mL) alone or LPS in combination with IgG-opsonized erythrocytes (E-IgG). Six hours after the addition of stimuli total RNA was isolated and used to carry out competitive RT-PCR. The ratio of the intensity of competitor (top band in each reaction) to that of the wild type (bottom band in each reaction) for the amplification reaction was used to determine cDNA levels. cDNAs were resolved on a 2% ethidium-stained agarose gel and normalized for HPRT intensities. Normalized cDNAs were then amplified in the presence of decreasing concentrations of competitor (PQRS), using primers for IL-12 p40. The concentration of the experimental cDNA is represented by the equivalent intensities of competitor and wild-type bands. The fold decrease in IL-12 (p40) levels between macrophages exposed to LPS or LPS in combination with E-IgG can be determined by taking the ratio of their equivalence points.

IFN-γ-mediated STAT1 activation [58]. This observation is consistent with previous studies which have shown that IgG immune complexes can inhibit IFN-γ-mediated STAT1 phosphorylation in human monocytes [59]. We have shown a correlation between the inhibition of IL-12 production and alterations in calcium influxes [57]. Macrophages from FcR γ-chain-deficient mice, which were incapable of fluxing calcium after FcγR ligation, were unable to suppress IL-12 production after FcγR ligation. Furthermore, the down-regulation of IL-12 by receptor ligation could be mimicked by experimentally fluxing calcium into macrophages using the calcium ionophore, ionomycin [57]. Finally, a macrophage-like cell line transfected with a luciferase construct driven by the IL-12 p40 promoter responded not only to receptor ligation but also to ionomycin treatment by reducing luciferase activity (**Fig. 3**). The calcium fluxes that we observe after receptor ligation may mediate a direct effect on gene transcription, as has been observed in other systems [60], or they may exert an indirect effect by altering levels of other intracellular products. Several investigators have recently correlated a decrease in IL-12 production with increased intracellular cAMP [61, 62]. Agonists of the β_2 adrenergic receptors [61] and prostaglandin E_2 (PGE_2) [62] both inhibit IL-12 biosynthesis and both increase intracellular cAMP. In our initial studies, the down-regulation of IL-12 induced by receptor ligation was not affected by experimentally manipulating cAMP with either dibutyryl cAMP or 2',5'-dideoxyadenosine to either increase or decrease, respectively, intracellular cAMP. We have performed electrophoretic mobility shift assays to identify alterations in the binding of specific transcription complexes to the IL-12 p40 promoter. The ligation of FcγR causes a dramatic decrease in the binding of the F1 complex to the Ets site of the IL-12 promoter. Ligation also diminishes NF-κB binding to its site by approximately half (unpublished observations). Thus, despite the fact that receptor ligation specifically diminishes IL-12 production, the decrease in transcription appears to be shared by both p40 and p35, and transcription factors, such as NF-κB, that are common to other cytokines may also be affected to some extent. Further studies on the mechanism of IL-12 suppression after receptor ligation, and a careful examination of the precise roles of protein phosphorylation, calcium alterations, and cAMP levels are warranted.

We have recently described a second way in which IL-12 is down-modulated after phagocytic receptor ligation [63]. This second mechanism is distinct from the first mechanism in two important ways. First, it is not a direct effect on IL-12 gene transcription, but rather is an indirect effect dependent on the production of the inhibitory cytokine IL-10. Second, this effect is not shared by all of the phagocytic receptors, but rather is specific to one of the FcγR. We have shown that the ligation of macrophage FcγR dramatically enhances IL-10 production in response to low amounts of LPS. Macrophages exposed to low (subnanogram) levels of LPS *in vitro* make insignificant amounts of IL-10 unless their FcγR are ligated, in which case they produce large amounts of IL-10 (**Fig. 4A**). The IL-10 that is produced by macrophages after FcγR ligation is sufficient to completely inhibit the production of IL-12 p70 by macrophages maximally stimulated with IFN-γ/LPS (Fig. 4B) [63]. This

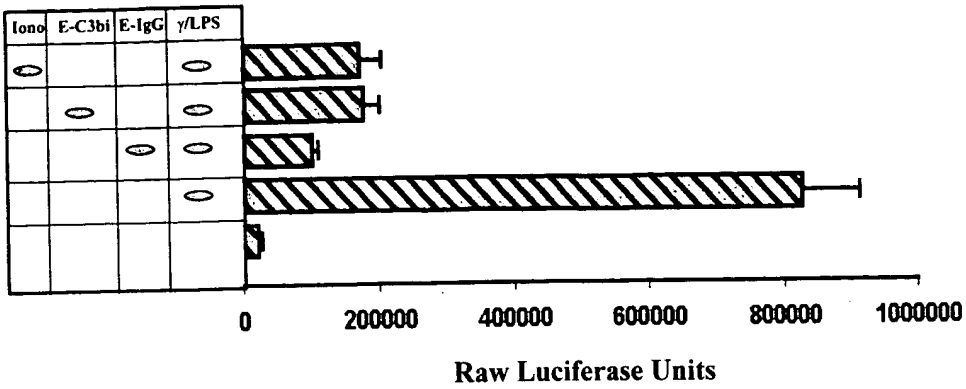

Fig. 3. Effect of receptor ligation on the IL-12 p40 promoter activity in RAW264.7 cells. RAW264.7 cells stably transfected with a human IL-12 p40 promoter-luciferase reporter construct were stimulated with IFN-γ (1000 U/mL) + LPS (1 μg/mL) alone or IFN-γ + LPS in combination with either IgG-opsonized erythrocytes (E-IgG), complement-opsonized erythrocytes (E-C3bi), or ionomycin (Iono) (5 μM). Eight hours after LPS stimulation, cells were harvested and total lysates prepared, and luciferase activity was assayed. Erythrocytes were added at a 20:1 ratio. Determinations were performed in triplicate and values are expressed as the mean ± SD.

observation is consistent with the recent report of Berger et al., who have shown that adding immune complexes to stimulated macrophages results in a decrease in IL-12 production and an increase in IL-10 [64]. Using macrophages from gene knockout mice, we have tentatively identified the FcγRI as the FcγR subtype responsible for the up-regulation of IL-10 production [63]. The stimulation of IL-10 production by specifically ligating the FcγRI with IgG3-opsonized erythrocytes [65] is consistent with the FcγRI-promoting macrophage IL-10 production (data not shown).

These observations implicate the FcγRI as an anti-inflammatory receptor by virtue of its ability to induce IL-10 production. Using transgenic mice, Heijnen et al. have shown that FcγRI is specifically up-regulated on inflammatory macrophages [66]. The observation that FcγRI ligation leads to IL-10 production would predict that targeting proinflammatory molecules to the FcγRI on macrophages will result in a decreased inflammatory response, characterized by diminished IL-12 and increased IL-10. This is exactly what we observed when we compared the cytokine response to LPS with IgG-opsonized LPS. IgG-opsonized LPS induces more IL-10 than LPS, but it induces very little IL-12 [63]. The observation that FcγRI co-ligation can increase IL-10 production is consistent with several previously published observations. Immune complexes have been shown to inhibit the *in vivo* clearance of *L. monocytogenes* via an IL-10-dependent mechanism [67]. The *in vitro* inhibition of macrophage tumoricidal and cytotoxic activity [68, 69] by immune complexes may be due to alterations in cytokine production after FcγR ligation.

These studies demonstrate that ligation of phagocytic receptors on monocytes and macrophages can down-modulate IL-12 production. These results would not have been predicted from the work of several groups, which demonstrates that a number of different microorganisms, including *Cryptococcus* [70], *Histoplasma* [71], and *Mycobacterium* [24], all induce IL-12 after their interaction with macrophages. Each of these organisms is taken up by receptor-mediated phagocytosis, and in all three cases, the complement receptors have been implicated in this process. How, then, can these organisms induce IL-12 production in the face of the strong inhibitory influences that have been observed by several laboratories after receptor ligation with defined particles? The complete answer to this paradox is not yet available, but several possible explanations exist. The first is that in addition to the inhibitory receptors discussed in this section, such as the phagocytic receptors, there are also IL-12 stimulatory receptors. Several of these stimulatory receptors have already been identified. CD40-CD40L interactions, CD14-LPS interactions, and the binding of hyaluronic acid to CD44 have all been shown to induce IL-12 [4, 13, 14]. The ligation of multiple IL-12-stimulatory receptors by microbes may render macrophages more resistant to IL-12 down-modulation after phagocytic receptor ligation. Another possible explanation lies in the potential heterogeneity of the IL-12-secreting cells. Mature macrophages, for example, exhibit quantitative differences in phagocytic receptor expression, and may therefore regulate IL-12 differently than do immature cells. Similarly, dendritic cells may be a primary source of IL-12 that is not as susceptible to down-regulation by phagocytic receptor ligation. All of these possible explanations will require further study before this apparent paradox can be explained.

PHARMACOLOGICAL METHODS TO DECREASE IL-12 PRODUCTION

Several compounds have been described that can inhibit IL-12 production when added to macrophages. These compounds include endogenous molecules made by macrophages themselves or a number of pharmacological compounds that are being tested as potential anti-inflammatory reagents. The three most well-described endogenous inhibitors of IL-12 biosynthesis are IL-10, transforming growth factor β (TGF-β), and PGE_2. These molecules are often made in response to the same stimuli that induce IL-12. All three of these compounds inhibit IL-12 production when added to macrophages before or simultaneous with proinflammatory stimuli. The inhibition by all three compounds occurs primarily at the level of transcription, and both the IL-12 p40 and p35 subunits appear to be affected.

The inhibitory effect of PGE_2 on IL-12 biosynthesis occurs

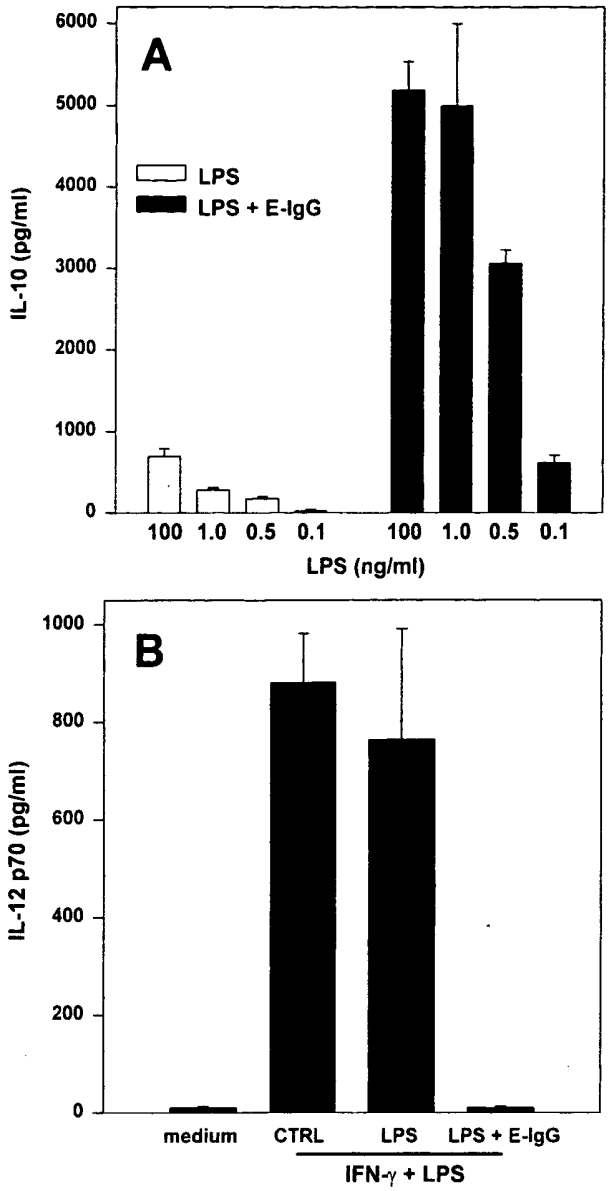

Fig. 4. LPS-induced IL-10 production is enhanced after FcγR ligation and is capable of suppressing IL-12 induction. (A) Bone marrow-derived macrophages were exposed to either LPS alone, at the concentrations indicated, or LPS in combination with IgG-opsonized erythrocytes (E-IgG). After 24 h, the supernatant was harvested and IL-10 levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the mean ± SD. (B) Supernatants from bone marrow-derived macrophages exposed to either LPS (0.5 ng/mL) or LPS (0.5 ng/mL) in combination with E-IgG were harvested after 24 h and filtered through a 0.2-μm filter. These supernatants were diluted 1:3 with medium and added to bone marrow-derived macrophages that had been primed with IFN-γ (100 U/mL) for 10 h, and then immediately treated with LPS (50 ng/mL). Parallel monolayers of macrophages were either left untreated (medium) or IFN-γ primed and then treated with LPS (CTRL). After 24 h, the supernatant was harvested and IL-12 p70 levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the mean ± SD.

by both direct and indirect mechanisms. The inhibition of IL-12 production by PGE_2 [62] correlates with the ability of PGE_2 to increase intracellular cAMP. The increase in intracellular cAMP induced by PGE_2 increases the production of IL-10, which inhibits IL-12 production. However, the inhibitory effect of PGE_2 is not dependent on IL-10 because neutralizing anti-IL-10 antibodies did not abrogate the inhibitory effect of PGE_2 [62]. The inverse correlation between intracellular cAMP and IL-12 production has also been established with agonists of the β_2 adrenergic receptors. These compounds are potent inhibitors of IL-12 [61]. This inhibition occurred at the level of transcription of both p35 and p40, and did not require protein synthesis. The decrease in IL-12 transcription by β_2 adrenergic agonists could be mimicked by pharmacological inducers of cAMP. It is important to note that the addition of β_2 adrenergic agonists to antigen-presenting cells could inhibit a Type 1 immune response by inhibiting the production of IL-12 [61]. 1,25-Dihydroxyvitamin D_3 is another naturally occurring compound that is able to inhibit IL-12 production when added to macrophages [72]. This inhibition affects both p35 and p40 and occurs at the level of gene transcription [72]. The inhibition of IL-12 biosynthesis by 1,25-dihydroxyvitamin D_3 is dependent on the presence of nuclear vitamin D_3 receptors and retinoid X receptors. Using gel shift analysis, a role for the NF-κB half site of the IL-12 p40 promoter has been established in this inhibition. The corticosteroid dexamethasone has also been shown to be capable of suppressing IL-12 production by human monocytes [73, 74]. Dexamethasone-mediated suppression of IL-12 occurs at the mRNA level for both the p40 and p35 genes [74]. Dexamethasone has also been shown to enhance IL-10 production by monocyte [73, 74]. Dexamethasone was still capable of inhibiting IL-12 production even in the presence of neutralizing anti-IL-10 antibodies, suggesting that dexamethasone does not rely on IL-10 production to suppress IL-12 [74]. Type I IFNs (IFN-α and -β) have been implicated in the inhibition of IL-12 during viral infections. IFN-α/β inhibited the *in vitro* production of IL-12 by both murine splenic leukocytes and by human dendritic cells [75, 76]. This inhibition was specific because the production of other proinflammatory cytokines, including TNF-α and IL-6, were not inhibited by IFN-α [75]. The IFN-α/β-mediated suppression of dendritic cell IL-12 production was correlated with an inhibition of Th1 development [76].

There are a number of divergent exogenous compounds that can also regulate IL-12 production. Pentoxifylline, intravenous immune globulin (IVIG), and thalidomide are examples of compounds that may have utility as anti-inflammatory therapeutics by virtue of their ability to inhibit IL-12 production. Pentoxifylline is a methylxanthine derivative that has been shown to have immunomodulatory activity. It is being used to treat immune-mediated disorders, such as rheumatoid arthritis and multiple sclerosis [77, 78]. The mechanism of action of pentoxifylline is not fully understood but recent *in vitro* studies have shown that the addition of pentoxifylline to monocytes can decrease IL-12 p40 and p35 mRNA production in response to inflammatory stimuli [79]. Pentoxifylline also increased the production of IL-10 and PGE_2, but the induction of these inhibitory compounds was not required for the inhibition of IL-12 by pentoxifylline. Similar to that of pentoxifylline, the immunosuppressive activity of thalidomide has been at least partially attributed to its ability to inhibit IL-12 [80]. Unlike that of pentoxifylline, however, the inhibition of IL-12 by thalidomide appeared to occur by posttranscriptional mechanisms. The inhibition of IL-12 by thalidomide might explain its

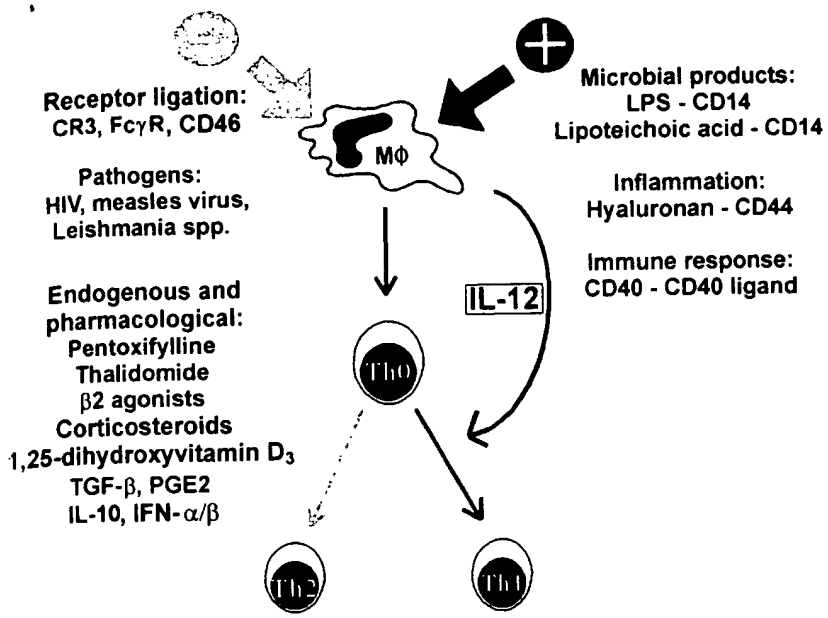

Fig. 5. Schematic representation of positive and negative regulators of macrophage IL-12 production.

potential utility in treating a number of autoimmune disorders. IVIG is another compound that is being used with increasing frequency as a nonspecific anti-inflammatory compound to treat autoimmune and systemic inflammatory disorders, including Kawasaki disease and idiopathic thrombocytopenic purpura [81]. We have recently treated murine macrophages *in vitro* with high doses of IVIG. These macrophages exhibit a marked decrease in IL-12 production with a coordinate increase in IL-10 (unpublished observations). This reciprocal alteration in cytokine production may be dependent on FcγR ligation. Our observations that IVIG can alter cytokine levels confirm previous observations by others [82].

In summary, several inhibitors of IL-12 production have recently been identified. These inhibitors range from receptor ligands to pharmacological compounds. Many of these inhibitory compounds share the following characteristics. Their inhibition is relatively specific to IL-12, and in many cases, p40 and p35 are coordinately affected. Protein synthesis is generally not required for the down-modulation but additional mechanisms that depend on the synthesis of inhibitory compounds, such as IL-10, can exert additive or synergistically inhibitory effects. In several instances a correlation between either calcium fluxes or cAMP have been observed, but in none of these examples thus far has cause and effect been definitively established. Additional studies on the induction of IL-12 will be required before studies to unravel its down-regulation will be possible.

SUMMARY

The production of IL-12 by antigen-presenting cells has enormous potential to influence the ensuing immune response. IL-12 elicits the production of IFN-γ from T cells and can skew the immune response toward a Th1 response (**Fig. 5**). By regulating the production of IL-12, this deviation toward a Th1-type immune response can be averted. The addition of exogenous pharmacological regulators of IL-12 have the potential to deviate the immune response away from a Th1-type immune response. The ligation of phagocytic receptors on antigen-presenting cells by antigen can exert a similar effect by directly down-regulating IL-12. Targeting antigen to the Fcγ receptors should be particularly effective in this respect because ligation of this receptor not only prevents IL-12 production but it also induces the production of IL-10 by inflammatory macrophages. The production of IL-10 in the presence of little or no IL-12 should predispose antigen-reactive T cells to mount a Th2-type immune response. The implication from these observations is that targeting antigen to different receptors on antigen-presenting cells can result in a fundamentally different immune response to that antigen.

ACKNOWLEDGMENTS

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REFERENCES

1. Wolf, S. F., Temple, P. A., Kobayashi, M., Young, D., Dicig, M., Lowe, L., Dzialo, R., Fitz, L., Ferenz, C., Hewick, R. M., Kelleher, K., Herrmann, S. H., Clark, S. C., Azzoni, L., Chan, S. H., Trinchieri, G., Perussia, B. (1991) Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J. Immunol. 146, 3074–3081.
2. Gubler, U., Chua, A. O., Schoenhaut, D. S., Dwyer, C. M., McComas, W., Motyka, R., Nabavi, N., Wolitzky, A. G., Quinn, P. M., Familletti, P. C., Gately, M. K. (1991) Coexpression of two distinct genes is required to

generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc. Natl. Acad. Sci. USA 88, 4143–4147.
3. Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chan, S., Loudon, R., Sherman, F., Perussia, B., Trinchieri, G. (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biological effects on human lymphocytes. J. Exp. Med. 170, 827–839.
4. D'Andrea, A., Rengaraju, M., Valiante, N. M., Chehimi, J., Kubin, M., Aste, M., Chan, S. H., Kobayashi, M., Young, D., Nickbarg, E., Chizzonite, R., Wolf, S. F., Trinchieri, G. (1992) Production of natural killer cell stimulatory factor (NKSF/IL-12) by peripheral blood mononuclear cells. J. Exp. Med. 176, 1387–1398.
5. Macatonia, S. E., Hosken, N. A., Litton, M., Vieira, P., Hsieh, C. S., Culpepper, J. A., Wysocka, M., Trinchieri, G., Murphy, K. M., O'Garra, A. (1995) Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4$^+$ T cells. J. Immunol. 154, 5071–5079.
6. Cassatella, M. A., Meda, L., Gasperini, S., D'Andrea, A., Ma, X., Trinchieri, G. (1995) Interleukin-12 production by human polymorphonuclear leukocytes. Eur. J. Immunol. 25, 1–5.
7. Cleveland, M. G., Gorham, J. D., Murphy, T. L., Tuomanen, E., Murphy, K. M. (1996) Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect. Immun. 64, 1906–1912.
8. Oswald, I. P., Dozois, C. M., Petit, J. F., Lemaire, G. (1997) Interleukin-12 synthesis is a required step in trehalose dimycolate-induced activation of mouse peritoneal macrophages. Infect. Immun. 65, 1364–1369.
9. Camargo, M. M., Almeida, I. C., Pereira, M. E., Ferguson, M. A., Travassos, L. R., Gazzinelli, R. T. (1997) Glycosylphosphatidylinositol-anchored mucin-like glycoproteins isolated from *Trypanosoma cruzi* trypomastigotes initiate the synthesis of proinflammatory cytokines by macrophages. J. Immunol. 158, 5890–5901.
10. Grunvald, E., Chiaramonte, M., Hieny, S., Wysocka, M., Trinchieri, G., Vogel, S. N., Gazzinelli, R. T., Sher, A. (1996) Biochemical characterization and protein kinase C dependency of monokine-inducing activities of *Toxoplasma gondii*. Infect. Immun. 64, 2010–2018.
11. Skeiky, Y. A., Guderian, J. A., Benson, D. R., Bacelar, O., Carvalho, E. M., Kubin, M., Badaro, R., Trinchieri, G., Reed, S. G. (1995) A recombinant Leishmania antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin-12. J. Exp. Med. 181, 1527–1537.
12. Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M. D., Silverman, G. J., Lotz, M., Carson, D. A., Raz, E. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 273, 352–354.
13. Hodge-Dufour, J., Noble, P. W., Horton, M. R., Bao, C., Wysoka, M., Burdick, M. D., Strieter, R. M., Trinchieri, G., Puré, E. (1997) Induction of IL-12 and chemokines by hyaluronan requires adhesion-dependent priming of resident but not elicited macrophages. J. Immunol. 159, 2492–2500.
14. Shu, U., Kiniwa, M., Wu, C. Y., Maliszewski, C., Vezzio, N., Hakimi, J., Gately, M., Delespesse, G. (1995) Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. Eur. J. Immunol. 25, 1125–1128.
15. Koch, F., Stanzl, U., Jennewein, P., Janke, K., Heufler, C., Kampgen, E., Romani, N., Schuler, G. (1996) High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. J. Exp. Med. 184, 741–746.
16. Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., Alber, G. (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J. Exp. Med. 184, 747–752.
17. Trinchieri, G., Gerosa, F. (1996) Immunoregulation by interleukin-12. J. Leukoc. Biol. 59, 505–511.
18. Hsieh, C.-S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., Murphy, K. M. (1993) Development of Th1 CD4$^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science 260, 547–549.
19. Manetti, R., Parronchi, P., Giudizi, M. G., Piccini, M.-P., Maggi, E., Trinchieri, G., Romagnani, S. (1993) Natural killer cell stimulatory factor (NKSF/IL-12) induces Th1-type specific immune responses and inhibits the development of IL-4 producing Th cells. J. Exp. Med. 177, 1199–1204.
20. Magram, J., Connaughton, S. E., Warrier, R. R., Carvajal, D. M., Wu, C. Y., Ferrante, J., Stewart, C., Sarmiento, U., Faherty, D. A., Gately, M. K. (1996) IL-12-deficient mice are defective in IFN-γ production and type 1 cytokine responses. Immunity 4, 471–481.
21. Chan, S. H., Perussia, B., Gupta, J. W., Kobayashi, M., Pospisil, M., Young, H. A., Wolf, S. F., Young, D., Clark, S. C., Trinchieri, G. (1991) Induction of IFN-γ production by natural killer cell stimulatory factor (NKSF): characterization of the responder cells and synergy with other inducers. J. Exp. Med. 173, 869–879.
22. Heinzel, F. P., Schoenhaut, D. S., Rerko, R. M., Rosser, L. E., Gately, M. K. (1993) Recombinant interleukin-12 cures mice infected with *Leishmania major*. J. Exp. Med. 177, 1505–1512.
23. Sypek, J. P., Chung, C. L., Mayor, S. E. H., Subramanyam, J. M., Goldman, S. J., Sieburth, D. S., Wolf, S. F., Schaub, R. G. (1993) Resolution of cutaneous leishmaniasis: interleukin-12 initiates a protective T helper type 1 immune response. J. Exp. Med. 177, 1797–1802.
24. Cooper, A. M., Roberts, A. D., Rhoades, E. R., Callahan, J. E., Getzy, D. M., Orme, I. M. (1995) The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. Immunology 84, 423–432.
25. Gazzinelli, R. T., Wysocka, M., Hayasi, S., Denkers, E. Y., Hieny, S., Caspar, P., Trinchieri, G., Sher, A. (1994) Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii*. J. Immunol. 153, 2533–2543.
26. Mattner, F., Magram, J., Ferrante, J., Launois, P., Dipadova, K., Behin, R., Gately, M. K., Louis, J. A., Alber, G. (1996) Genetically resistant mice lacking interleukin-12 are susceptible to infection with *Leishmania major* and mount a polarized Th2 cell response. Eur. J. Immunol. 26, 1553–1559.
27. Scharton-Kerston, T., Afonso, L. C. C., Wysocka, M., Trinchieri, G., Scott, P. (1995) Interleukin-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. J. Immunol. 154, 5320–5330.
28. Tripp, C. S., Gately, M. K., Hakimi, J., Ling, P., Unanue, E. R. (1994) Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice. Reversal by interferon-γ. J. Immunol. 152, 1883–1887.
29. Afonso, L. C., Scharton, T. M., Vieira, L. Q., Wysocka, M., Trinchieri, G., Scott, P. (1994) The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. Science 263, 235–237.
30. Wysocka, M., Marek, M., Vieira, L. Q., Ozmen, L., Garotta, G., Scott, P., Trinchieri, G. (1995) Interleukin-12 is required for interferon-γ production and lethality in lipopolysaccharide-induced shock in mice. Eur. J. Immunol. 25, 672–676.
31. Trembleau, S., Penna, G., Bosi, E., Mortara, A., Gately, M. K., Adorini, L. (1995) Interleukin-12 administration induces T helper type 1 cells and accelerates autoimmune diabetes in NOD mice. J. Exp. Med. 181, 817–821.
32. Leonard, J. P., Waldburger, K. E., Goldman, S. J. (1995) Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin-12. J. Exp. Med. 181, 381–386.
33. Germann, T., Szeliga, J., Hess, H., Storkel, S., Podlaski, F. J., Gately, M. K., Schmitt, E., Rude, E. (1995) Administration of interleukin 12 in combination with type II collagen induces severe arthritis in DBA/1 mice. Proc. Natl. Acad. Sci. USA. 92, 4823–4827.
34. Ma, X., Neurath, M., Gri, G., Trinchieri, G. (1997) Identification and characterization of a novel Ets-2-related nuclear complex implicated in the activation of the human interleukin-12 p40 gene promoter. J. Biol. Chem. 272, 10389–10395.
35. Murphy, T. L., Cleveland, M. G., Kulesza, P., Magram, J., Murphy, K. M. (1995) Regulation of interleukin 12 p40 expression through an NF-κB half-site. Mol. Cell Biol. 15, 5258–5267.
36. Gri, G., Savio, D., Trinchieri, G., Ma, X. (1998) Synergistic regulation of the human interleukin-12 p40 promoter by NFκB and Ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. J. Biol. Chem. 273, 6431–6438.
37. Plevy, S. E., Gemberling, J. H., Hsu, S., Dorner, A. J., Smale, S. T. (1997) Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. Mol. Cell. Biol. 17, 4572–4588.
38. Hayes, M. P., Wang, J., Norcross, M. A. (1995) Regulation of interleukin-12 expression in human monocytes: selective priming by IFN-γ of LPS inducible p35 and p40 genes. Blood 86, 646–650.
39. Snijders, A., Hilkens, C. M., van der Pouw Kraan, T. C., Engel, M., Aarden, L. A., Kapsenberg, M. L. (1996) Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. J. Immunol. 156, 1207–1212.
40. Hayes, M. P., Murphy, F. J., Burd, P. R. (1998) Interferon-γ-dependent inducible expression of the human IL-12 p35 gene in monocytes initiates from a TATA-containing promoter distinct from the CpG-rich promoter active in Epstein-Barr virus-transformed lymphoblastoid cells. Blood 91, 4645–4651.
41. Tone, Y., Thompson, A., Babik, J. M., Nolan, K. F., Tone, M., Raven, C., Waldmann, H. (1996) Structure and chromosomal location of the mouse interleukin 12 p35 and p40 subunit genes. Eur. J. Immunol. 26, 1222–1227.
42. Yashimoto, T., Kojima, K., Funakoshi, T., Endo, Y., Fujita, T., Nariuchi, H. (1996) Molecular cloning and characterization of murine IL-12 genes. J. Immunol. 156, 1082–1088.

43. Ma, X., Chow, J. M., Gri, G., Carra, G., Gerosa, F., Wolf, S. F., Dzialo, R., Trinchieri, G. (1996) The interleukin-12 p40 gene promoter is primed by interferon-γ in monocytic cells. J. Exp. Med. 183, 147–157.
44. Munder, M., Mallo, M., Eichmann, K., Modolell, M. (1998) Murine macrophages secrete interferon γ upon combined stimulation with interleukin (IL)-12 and IL-18: a novel pathway of autocrine macrophage activation. J. Exp. Med. 187, 2103–2108.
45. Carrera, L., Gazzinelli, R. T., Badolato, R., Hieny, S., Muller, W., Kuhn, R., Sacks, D. L. (1996) Leishmania promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. J. Exp. Med. 183, 515–526.
46. Belkaid, Y., Butcher, B., Sacks, D. L. (1998) Analysis of cytokine production by inflammatory mouse macrophages at the single-cell level: selective impairment of IL-12 induction in Leishmania-infected cells. Eur. J. Immunol. 28, 1389–1400.
47. Weinheber, N., Wolfram, M., Harbecke, D., Aebischer, T. (1998) Phagocytosis of *Leishmania mexicana* amastigotes by macrophages leads to a sustained suppression of IL-12 production. Eur. J. Immunol. 28, 2467–2477.
48. Karp, C. L., Wysocka, M., Wahl, L. M., Ahearn, J. M., Cuomo, P. J., Sherry, B., Trinchieri, G., Griffin, D. E. (1996) Mechanism of suppression of cell-mediated immunity by measles virus. Science 273, 228–231.
49. Chehimi, J., Starr, S. E., Frank, I., D'Andrea, A., Ma, X., MacGregor, R. R., Sennelier, J., Trinchieri, G. (1994) Impaired interleukin 12 production in human immunodeficiency virus-infected patients. J. Exp. Med. 179, 1361–1366.
50. Chougnet, C., Wynn, T. A., Clerici, M., Landay, A. L., Kessler, H. A., Rusnak, J., Melcher, G. P., Sher, A., Shearer, G. M. (1996) Molecular analysis of decreased interleukin-12 production in persons infected with human immunodeficiency virus. J. Infect. Dis. 174, 46–53.
51. Reiner, S. L., Zheng, S., Wang, Z. E., Stowring, L., Locksley, R. M. (1994) Leishmania promastigotes evade interleukin-12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from $CD4^+$ T cells during the initiation of infection. J. Exp. Med. 179, 447–456.
52. Mosser, D. M., Springer, T. S., Diamond, M. S. (1992) Leishmania promastigotes require opsonic complement to bind to the human leukocyte integrin Mac-1 (CD11b/CD18). J. Cell Biol. 116, 511–520.
53. Tamashiro, V. G., Perez, H. H., Griffin, D. E. (1987) Prospective study of the magnitude and duration of changes in tuberculin reactivity during uncomplicated and complicated measles. Pediatr. Infect. Dis. J. 6, 451–454.
54. Clerici, M., Shearer, G. M. (1993) A TH1 → TH2 switch is a critical step in the etiology of HIV infection. Immunol. Today 14, 107–111.
55. Stoiber, H., Clivio, A., Dierich, M. P. (1997) Role of complement in HIV infection. Annu. Rev. Immunol. 15, 649–674.
56. Gazzinelli, R. T., Bala, S., Stevens, R., Baseler, M., Wahl, L., Kovacs, J., Sher, A. (1995) HIV infection suppresses Type 1 lymphokine and IL-12 responses to *Toxoplasma gondii* but fails to inhibit the synthesis of other parasite-induced monokines. J. Immunol. 155, 1565–1574.
57. Sutterwala, F. S., Noel, G. J., Clynes, R., Mosser, D. M. (1997) Selective suppression of IL-12 induction after macrophage receptor ligation. J. Exp. Med. 185, 1977–1985.
58. Marth, T., Kelsall, B. L. (1997) Regulation of interleukin-12 by complement receptor 3 signaling. J. Exp. Med. 185, 1987–1995.
59. Feldman, G. M., Chuang, E. J., Finbloom, D. S. (1995) IgG immune complexes inhibit IFN-γ-induced transcription of the FcγRI gene in human monocytes by preventing the tyrosine phosphorylation of the p91 (Stat1) transcription factor. J. Immunol. 154, 318–325.
60. Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C., Healy, J. I. (1997) Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. Nature 386, 855–858.
61. Panina-Bordignon, P., Mazzeo, D., Lucia, P. D., D'Ambrosio, D., Lang, R., Fabbri, L., Self, C., Sinigaglia, F. (1997). β_2 agonists prevent Th1 development by selective inhibition of interleukin-12. J. Clin. Invest. 100, 1513–1519.
62. Tineke, C., van der Pouw Kraan, T. M., Boeije, L. C. M., Smeenk, R. J. T., Wijdens, J., Aarden, L. A. (1995) Prostaglandin E_2 is a potent inhibitor of human IL-12 production. J. Exp. Med. 181, 775–779.
63. Sutterwala, F. S., Noel, G. J., Salgame, P., Mosser, D. M. (1998) Reversal of proinflammatory responses by ligating the macrophage FcγRI. J. Exp. Med. 188, 217–222.
64. Berger, S., Chandra, R., Ballo, H., Hildenbrand, R., Stutte, H. J. (1997) Immune complexes are potent inhibitors of interleukin-12 secretion by human monocytes. Eur. J. Immunol. 27, 2994–3000.
65. Gavin, A. L., Barnes, N., Dijstelbloem, H. M., Hogarth, P. M. (1998) Identification of the mouse IgG3 receptor: implications for antibody effector functions at the interface between innate and acquired immunity. J. Immunol. 160, 20–23.
66. Heijnen, I. A. F., van Vugt, M. J., Fanger, N. A., Graziano, R. F., de Wit, T. P. M., Hofhuis, F. M. A., Guyre, P. M., Capel, P. J. A., Verbeek, J. S., van de Winkle, J. G. (1996) Antigen targeting to myeloid-specific human FcγRI/CD64 triggers enhanced antibody responses in transgenic mice. J. Clin. Invest. 97, 331–338.
67. Tripp, C. S., Beckerman, K. P., Unanue, E. R. (1995) Immune complexes inhibit antimicrobial responses through interleukin-10 production: effects in severe combined immunodeficient mice during Listeria infection. J. Clin. Invest. 95, 1628–1634.
68. Esparza, I., Green, R., Schreiber, R. D. (1983) Inhibition of macrophage tumoricidal activity by immune complexes and altered erythrocytes. J. Immunol. 131, 2117–2121.
69. Virgin, H. W., Kurt-Jones, E. A., Wittenberg, G. F., Unanue, E. R. (1985) Immune complex effects on murine macrophages: II. Immune complex effects on activated macrophages cytotoxicity, membrane IL-1 and antigen presentation. J. Immunol. 135, 3744–3749.
70. Harrison, T. S., Levitz, S. M. (1996) Role of IL-12 in peripheral blood mononuclear cell responses to fungi in persons with and without HIV infection. J. Immunol. 156, 4492–4497.
71. Zhou, P., Sieve, M. C., Bennett, J., Kwon-Chung, K. J., Tewari, R. P., Gazzinelli, R. T., Sher, A., Seder, R. A. (1995) IL-12 prevents mortality in mice infected with *Histoplasma capsulatum* through the induction of IFN-γ. J. Immunol. 155, 785–799.
72. D'Ambrosio, D., Cippitelli, M., Cocciolo, M. G., Mazzeo, D., Di Lucia, P., Lang, R., Sinigaglia, F., Panina-Bordignon, P. (1998) Inhibition of IL-12 production by 1,25-dihydroxyvitamin D_3: involvment of NFκB downregulation in transcriptional repression of the p40 gene. J. Clin. Invest. 101, 252–262.
73. Blotta, M. H., DeKruyff, R. H., Umetsu, D. T. (1997) Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in $CD4^+$ lymphocytes. J. Immunol. 158, 5589–5595.
74. Visser, J., van Boxel-Dezaire, A., Methorst, D., Brunt, T., de Kloet, E. R., Nagelkerken, L. (1998) Differential regulation of interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro. Blood 91, 4255–4264.
75. Cousens, L. P., Orange, J. S., Su, H. C., Biron, C. A. (1997) Interferon-α/β inhibition of interleukin 12 and interferon-γ production in vitro and endogenously during viral infection. Proc. Natl. Acad. Sci. USA. 94, 634–639.
76. McRae, B. L., Semnani, R. T., Hayes, M. P., van Seventer, G. A. (1998) Type I IFNs inhibit human dendritic cell IL-12 production and Th1 cell development. J. Immunol. 160, 4298–4304.
77. Maksymowych, W. P., Avina-Zubieta, A., Luong, M. H., Russell, A. S. (1995) An open study of pentoxifylline in the treatment of severe refractory rheumatoid arthritis. J. Rheumatol. 22, 625–629.
78. Rieckmann, P., Weber, F., Gunther, A., Martin, S., Bitsch, A., Broocks, A., Kitze, B., Weber, T., Borner, T., Poser, S. (1996) Pentoxifylline, a phosphodiesterase inhibitor, induces immune deviation in patients with multiple sclerosis. J. Neuroimmunol. 64, 193–200.
79. Moller, D. R., Wysocka, M., Greenlee, B. M., Ma, X., Wahl, L., Trinchieri, G., Karp, C. L. (1997) Inhibition of human interleukin-12 production by pentoxifylline. Immunology 91, 197–203.
80. Moller, D. R., Wysocka, M., Greenlee, B. M., Ma, X., Wahl, L., Flockhart, D. A., Trinchieri, G., Karp, C. L. (1997) Inhibition of IL-12 production by thalidomide. J. Immunol. 159, 5157–5161.
81. Ballow, M. (1997) Mechanisms of action of intravenous immune serum globulin in autoimmune and inflammatory diseases. J. Allergy Clin. Immunol. 100, 151–157.
82. Andersson, J., Skansen-Saphir, U., Sparrelid, E., Andersson, U. (1996) Intravenous immune globulin affects cytokine production in T lymphocytes and monocytes/macrophages. Clin. Exp. Immunol. 104, S10–S20.

Concise Review

Mechanism of Activation of the GM-CSF, IL-3, and IL-5 Family of Receptors

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ABSTRACT

The process of ligand binding leading to receptor activation is an ordered and sequential one. High-affinity binding of GM-CSF, interleukin 3 (IL-3), and IL-5 to their receptors induces a number of key events at the cell surface and within the cytoplasm that are necessary for receptor activation. These include receptor oligomerization, activation of tyrosine kinase activity, phosphorylation of the receptor, and the recruitment of SH2 (src-homology) and PTB (phosphotyrosine binding) domain proteins to the receptor. Such a sequence of events represents a recurrent theme among cytokine, growth factor, and hormone receptors; however, a number of very recent and interesting findings have identified unique features in this receptor system in terms of: A) how GM-CSF/IL-3/IL-5 bind, oligomerize, and activate their cognate receptors; B) how multiple biological responses such as proliferation, survival, and differentiation can be transduced from activated GM-CSF, IL-3, or IL-5 receptors, and C) how the presence of novel phosphotyrosine-independent signaling motifs within a specific cytoplasmic domain of β_C may be important for mediating survival and differentiation by these cytokines. This review does not attempt to be all-encompassing but rather to focus on the most recent and significant discoveries that distinguish the GM-CSF/IL-3/IL-5 receptor subfamily from other cytokine receptors. *Stem Cells 1998;16:301-313*

INTRODUCTION

The ability of multicellular organisms to coordinate cell proliferation, survival, and differentiation as well as a variety of other specialized cell functions is absolutely dependent on the capacity of different cell types to communicate. Among the vast numbers of different cells within the body, nature has designed an elegant solution to achieving cell-cell communication that does not require cell-cell contact through the production of cytokines and growth factors. A cytokine/growth factor has the capacity to relay information from a producer cell to a second responding cell located either in close proximity or at other sites in the body by virtue of binding to specific receptors on the surface of the cell. This in turn triggers a biochemical cascade inside the cell leading to varied biological responses such as motility, adhesion, growth, survival, and differentiation.

Central to an organism's need to meet hemopoietic demands and to mount an immune/inflammatory response is its ability to stimulate specific blood cell populations. GM-CSF, interleukin 3 (IL-3), and IL-5 are cytokines principally produced by activated T cells which exhibit pleiotropic activities, stimulating proliferation, survival, and differentiation of myeloid hemopoietic cells and the effector function of the terminally differentiated myeloid cells [1, 2]. GM-CSF receptors have been identified on most types of myeloid progenitors and on mature monocytes, neutrophils, eosinophils, basophils, and dendritic cells [2]. IL-3 receptors are present on early hemopoietic progenitor cells, on certain committed myeloid progenitors, eosinophils, and basophils, while IL-5 receptors have been shown to be expressed mainly on eosinophils [2]. Consistent with the pattern of receptor expression, GM-CSF and IL-3 can stimulate immature

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myelomonocytic cells in the hemopoietic system and cause differentiation of the granulocyte and macrophage populations [2]. In addition, GM-CSF and IL-3 are important for inducing the effector functions of these cells and hence contribute to the body's defense against microbial pathogens [1, 2]. IL-5 can chiefly stimulate production, survival, and granule release of eosinophils and participate in allergic reactions and anti-parasite responses [1, 2]. Experiments aimed at investigating the in vivo function of GM-CSF, IL-3, and IL-5 using a gene knockout approach would suggest that these cytokines are not essential in maintaining "steady-state" populations of granulocytes, macrophages, and eosinophils, but instead are central for the accelerated production of these cells in inflammation and infection. As such, they may be considered more as "reactive" cytokines required in emergency situations.

Apart from its proposed role in stimulated hemopoiesis and inflammatory responses, GM-CSF has also been implicated in cell transformation. The GM-CSF receptor has been identified in different types of cancer cells, including those of acute myeloid leukemia, chronic myeloid leukemia, juvenile myelomonocytic leukemia, melanoma, certain breast cancer cell lines, small-cell lung carcinoma, and the prostate [3, 4]. In all of these cells (with the apparent exception of some melanoma cell lines), both subunits of the GM-CSF receptor have been detected, suggesting that the receptor is capable of fully transducing GM-CSF signals and hence contributing to proliferative and/or survival functions.

The receptors for GM-CSF, IL-3, and IL-5 consist of a ligand-specific α subunit (GMRα, IL-3Rα, and IL-5Rα respectively) [5-7] and a common β subunit (β_C) [8]. The α subunit is the major ligand-binding subunit and on its own does not seem to transduce any of the biological activities ascribed to GM-CSF, IL-3 and IL-5 in hemopoietic cells [9, 10]. The β_C subunit, on the other hand, converts the ligand-bound α subunit to a high affinity state and is important for most, if not all, of the signaling [7, 8, 11]. Curiously, there are two β subunits in mice; a β_C which can be activated by GM-CSF/IL-3/IL-5, and the IL-3-specific β subunit, $\beta_{IL\text{-}3}$, which binds IL-3 with low affinity and only forms a high-affinity receptor with IL-3Rα [12, 13]. In terms of cross-reactivity, human GM-CSF does not bind to mouse GM-CSF receptors detectably; however, crosstalk between human and mouse receptors has been observed. FDCP1 cells that express endogenous mouse GM-CSF receptors and which are transfected with the human GMRα exhibit crosstalk between the receptor species [14]. This trans-species crosstalk phenomenon should be borne in mind for interpreting results, as proliferative and survival signals apparently mediated by human receptors may be confounded by the presence of endogenous mouse receptors.

Activation of the GM-CSF Family of Receptors: The Extracellular Story

GM-CSF, IL-3, and IL-5 Receptor Extracellular Structure

The specific receptor α subunits for GM-CSF, IL-3 and IL-5 as well as β_C are members of the type I cytokine receptor superfamily and contain conserved extracellular domains termed cytokine receptor modules (CRMs) [15]. Each CRM consists of two repeats of a fibronectin type III-like domain. These repeats carry two sets of conserved motifs typical of this family of receptors. The first repeat contains four cysteines with conserved spacing, while the second repeat contains a WSXWS motif. The homology between β_C and the growth hormone (GH) receptor has allowed the extracellular structure of β_C to be modeled from the crystal structure of the GH receptor [16, 17]. This model predicts that the four spatially conserved cysteines within the CRM of β_C will form disulfide bonds and are important for maintaining the structural integrity of β_C. In terms of the WSXWS motif, mutagenesis of this sequence in a number of cytokine receptors has been shown to disrupt cytokine binding and receptor activation. However, from the crystal structure of GH bound to the GH receptor, the WSXWS-equivalent motif does not appear to be directly involved in ligand binding [17]. Mutagenesis studies in both the GH receptor and the erythropoietin (EPO) receptor suggest that the WSXWS motif is important for correct folding of the extracellular domain of cytokine receptors [18, 19].

High-Affinity Binding

Reconstitution studies where both the GMRα or β_C are expressed at the cell surface indicate that GM-CSF first binds to GMRα with low affinity ($K_d \sim 5 \times 10^{-9}$M) and that this complex is converted to high affinity following recruitment of β_C ($K_d \sim 10^{-11}$M) [7, 8, 11]. Such an observation would imply that GM-CSF would have at least two binding interfaces, one that first binds GMRα and one that subsequently recruits and binds β_C to form a 1:1:1 complex of GM-CSF:GMRα:β_C. A model for such an interaction in illustrated in Figure 1. Since GM-CSF cannot detectably bind β_C directly in the absence of GMRα, it would also seem likely that there are domains within GMRα that cooperate with GM-CSF in binding β_C (Fig. 1). Mutagenesis of GM-CSF, GMRα, and β_C indicate that, in fact, there are likely to be two binding interfaces on GM-CSF important for the formation of a 1:1:1 complex. For low-affinity binding, residues centered on the fourth α helix of GM-CSF involving Asp112 are likely to be important for establishing an ionic interaction, possibly through Arg280 of GMRα [20, 21]. On the second binding interface, a single conserved glutamate residue located in the first α helix of GM-CSF, IL-3 and IL-5 is essential for high-affinity binding [22-24]. Mutants of this residue in GM-CSF (E21R) [23], IL-3

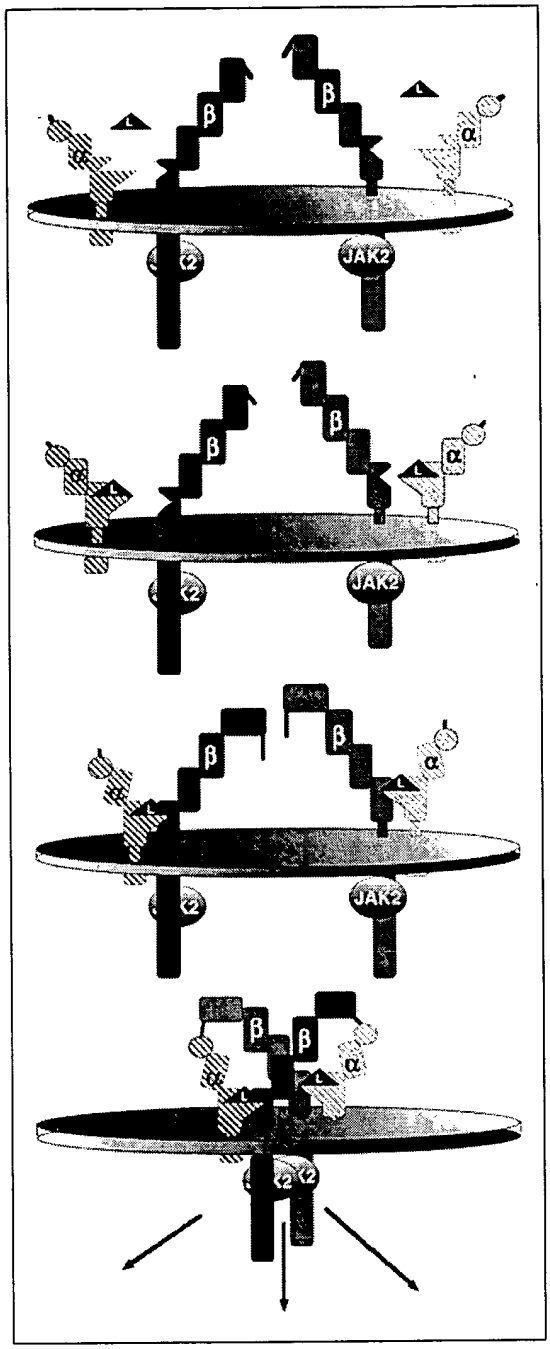

Figure 1. Model for the activation of the GM-CSF, IL-3, or IL-5 receptor. *The extracellular portion of β_C contains domains 1 to 4 (from the N-terminus) which are illustrated as rounded oblongs. The α-subunit contains three domains. Receptor activation is thought to occur by a stepwise process. GM-CSF, IL-3, and IL-5 (L) first bind the α-subunit with low affinity and this complex is converted to high affinity following recruitment of β_C to form a 1:1:1 complex of ligand:α:β_C. Dimerization of this complex, possibly through disulfide binding (shown as solid lines), forms a 2:2:2 complex that allows JAK2 activation and signaling.*

(E22R) [22], or IL-5 (E13Q) [24] abolish high-affinity binding and result in either functional antagonism (GM-CSF:E21R and IL-5:E13Q) or weak agonism (IL-3:E22R). On the other hand, substitution with a neutral amino acid at this position such as alanine (GM-CSF-E21A) resulted in weak agonism, indicating that a GM-CSF/GMRα complex may recruit β_C through a charge interaction involving Glu21 of GM-CSF and a positively charged amino acid on β_C [23]. The close association of ligand and β_C is also supported by the finding that GM-CSF can be cross-linked to β_C following receptor activation [8].

Identification of the residues in β_C that interact with the conserved glutamate of GM-CSF, IL-3, and IL-5 has been difficult, although there is evidence that amino acids in the membrane proximal domain 4 region of β_C are important (Fig. 1). Antibodies that bind this domain block high-affinity binding of GM-CSF (unpublished data). Moreover, mutagenesis of β_C indicates that residues Tyr365, His367 and Ile368 within domain 4 are important for GM-CSF and IL-5 high-affinity binding whereas Tyr421 is required for GM-CSF, IL-5, and IL-3 high-affinity binding [25-27]. However, identification of the precise amino acids within β_C that interact with the conserved glutamate of GM-CSF/IL-3/IL-5 awaits the crystallization of the ligand/receptor complex.

Receptor Dimerization and Activation

While type I transmembrane receptors vary considerably in terms of their structure, subunit composition, and the nature of ligand binding, it has been repeatedly shown that receptor dimerization is an obligatory event in receptor activation [28]. The GM-CSF/IL-3/IL-5 receptors are no exception to this general rule. However, while the formation of a 1:1:1 complex of GM-CSF:GMRα:β_C may represent a high-affinity binding complex, it is much less clear what the stoichiometry of the active receptor complex is. Studies using dominant-negative, chimeric, and mutant receptors as well as modeling studies would indicate that at least two β_C subunits are required for receptor activation and signaling and that the active GM-CSF receptor is composed of a 2:2:2 complex of GM-CSF:GMRα:β_C. A chimeric receptor consisting of an EPO receptor extracellular domain, which is known to dimerize in response to EPO, and a β_C cytoplasmic domain were examined for their ability to confer growth signals in response to EPO. Expression of these receptors in Ba/F3 cells resulted in EPO-dependent growth, indicating that β_C cytoplasmic-domain dimerization is sufficient for signaling [29]. In addition, GM-CSF was able to support the growth of cells coexpressing wild-type β_C and a chimeric receptor consisting of the GMRα extracellular domain and the β_C intracellular domain, further supporting the idea that dimerization of β_C intracellular

domains is sufficient for receptor activation [10]. In a reverse experiment, GM-CSF was not able to support cell growth in cells coexpressing wild-type GMRα and a chimeric receptor consisting of the extracellular domain of β_C and the cytoplasmic domain of GMRα suggesting that dimerization of β_C but not α chains is sufficient for receptor activation [10]. Two α chains nevertheless appear to be necessary for the formation of an active complex. Coexpression of a cytoplasmically truncated form of GMRα with wild-type GMRα and β_C in NIH 3T3 cells impairs cell proliferation and focus formation in response to GM-CSF [30].

Other studies have used chimeric receptors consisting of either the Fos or Jun leucine zippers in the extracellular domains and either the GMRα or β_C intracellular domains to investigate the stoichiometry of the active receptor. Heterodimerization of GMRα and β_C intracellular domains or homodimerization of β_C intracellular domains mediated by leucine zipper dimerization both promoted cytokine-independent growth in Ba/F3 cells [31]. While the ability of leucine zipper-mediated β_C intracellular homodimers to promote cell proliferation is in line with the above experiments examining the role of dimerization in receptor activation, the finding that leucine zipper-mediated heterodimerization of GMRα and β_C intracellular domains supported cell growth would imply that a 1:1:1 complex of GM-CSF:GMRα:β_C would also be capable of signaling. However, it is difficult to exclude the possibility in these experiments that, in addition to heterodimerization of GMRα and β_C intracellular domains, homodimers of β_C intracellular domains were also formed or that the endogenous mouse β is recruited into these complexes.

Overall, these data together with modeling studies, suggest a model in which the active receptor complex is formed following the dimerization of two 1:1:1 high-affinity complexes of GM-CSF:GMRα:β_C to form an activated receptor composed of a 2:2:2 complex (Fig. 1). In its simplest form, this model proposes that the active receptor complex would contain two β_C subunits; however, higher-order oligomeric complexes may also form functionally activated receptors.

A critical event in the production of an active receptor complex is not only its initial formation but also its subsequent stability. In the case of the GM-CSF/IL-3/IL-5 receptors, stability is likely to be promoted by a number of factors. First, there are likely to be domains within the 1:1:1 complex of GM-CSF:GMRα:β_C that are responsible for the recruitment and binding of a second 1:1:1 complex. In a model similar to that proposed by *Stahl* and *Yancopolous* for IL-6 binding, GM-CSF may have three binding interfaces: one that interacts with GMRα, one that interacts with β_C (as discussed above and shown in Fig. 1), and one that interacts with and recruits a second high-affinity GM-CSF:GMRα:β_C complex [32]. While this possibility remains to be tested, molecular modeling of the GM-CSF:GMRα:β_C complex using the crystal structure of GH bound to the GH receptor would suggest that an alternative mechanism for receptor dimerization may be at work [16, 17]. In terms of size, GM-CSF, IL-3, and IL-5 are smaller than the IL-6 family of cytokines. It would seem unlikely from these models that the smaller size of GM-CSF, IL-3, and IL-5 would permit a third binding interface for the recruitment of a second ligand:Rα:β_C complex as is proposed to occur for IL-6 [32]. Alternatively, we propose that high-affinity binding of GM-CSF, IL-3, or IL-5 and the formation of a ligand:Rα:β_C complex may create specific motifs or expose specific residues that mediate dimerization and the formation of a 2:2:2 complex of ligand:Rα:β_C. This is based on our observation that β_C contains, apart from the spatially conserved cysteines of the CRM, two additional cysteine residues, C86 and C91, that are conserved between mouse and human β_C and which are involved in the formation of a disulfide linkage with cysteines in the α subunit [33]. IL-3 stimulation of COS cells expressing IL-3Rα and either β_CC86A or β_CC91A resulted in high-affinity binding but no β_C tyrosine phosphorylation. While IL-3Rα/β_C disulfide-linked dimers are normally observed following ligand stimulation, these dimers were not detected following stimulation of cells expressing either β_CC86A or β_CC91A [33]. As proposed by *Stomski et al.* [33] and illustrated in Figure 1, these disulfide linkages most likely occur between two different ligand:Rα:β_C complexes and thus may be responsible for the dimerization and stability of an active 2:2:2 complex.

The conserved motif represented by Cys 86 and Cys 91 in human and mouse β_C is not found in related cytokine receptors and suggests an evolutionary conserved structural and functional basis for activation of the GM-CSF, IL-3, and IL-5 receptors. A second unique feature of this receptor subfamily is the demonstration that, in the case of the GM-CSF receptor, a proportion of these receptors are preassembled (before the addition of GM-CSF). We and others have shown that mutations of GMRα that abolish GM-CSF binding are compensated for when coexpressed with β_C [21, 34]. As β_C is unable to detectably bind GM-CSF in the absence of GMRα, this suggests that β_C is pre-associated with GMRα prior to the addition of GM-CSF. We have now established that a proportion of GM-CSF receptors are preassociated by showing that GMRα and β_C can be coimmunoprecipitated in the absence of GM-CSF from primary leukemic cells and myeloid cell lines, and in GM-CSF receptor transfected cells [35]. This is in direct contrast to the IL-3 and IL-5 receptors in which the α subunit and β_C associate only in the presence of IL-3 or IL-5, respectively [35]. The preformed GMRα:β_C complex may be responsible for the rapid association of GM-CSF to monocytes and eosinophils relative to IL-3 and IL-5. The kinetics of

association for GM-CSF binding to eosinophils and monocytes corresponds to two classes of receptors, a relatively slow one with association characteristics identical to those of IL-3 and IL-5, presumably representing ligand-induced receptors, and a second class which exhibits virtually instantaneous GM-CSF binding and possibly represents preformed receptors [35].

Interestingly, the preformed GMRα/β_C complex may be activated not only by GM-CSF but also by IL-3 or IL-5 [35]. The significance of this is unclear; however, it may provide a mechanism for regulating or increasing the diversity of cytokine signaling. As has been proposed in other receptor systems, heterodimerization of different members of a cytokine receptor family may allow the recruitment of different signaling molecules. For example, several members of the epidermal growth factor (EGF) receptor family, such as Neu, ErbB3 and the EGF receptor itself, have shown the potential to heterodimerize in response to EGF or a related ligand, heregulin [36]. In fact, the ability of EGF to generate diversity in signaling through heterodimerization of receptors has been demonstrated with respect to phosphatidylinositol 3-kinase (PI 3-kinase). Although EGF is known to activate PI 3-kinase activity in some cells, the EGF receptor does not appear to contain a motif for the recruitment of PI 3-kinase. However, ErbB3 contains a PI 3-kinase binding motif. EGF binding to the EGF receptor results in the recruitment and activation of ErbB3 which in turn allows PI 3-kinase activation [37]. Clearly, crosstalk between different receptors has the potential to increase the signaling repertoire for a receptor family and may account for some of the diverse biological functions attributed to GM-CSF, IL-3, and IL-5.

A preformed GM-CSF receptor complex and the indirect recruitment of β_C may also have implications pertinent to the cytokine receptor family at large, since they may explain crosstalk by other cytokine receptors and the universal expression of the GM-CSF receptor in the hemopoietic system. In murine cells, tyrosine phosphorylation of mouse β_C chains has been demonstrated in response to G-CSF [38] and EPO. Similarly, stem cell factor stimulation has been shown to result in cross-phosphorylation of mouse β chains on serine or threonine [39]. In the human system, EPO stimulation has been shown to unidirectionally cross-phosphorylate β_C in UT7 cells [40]. More recently, thrombopoietin has also been demonstrated to unidirectionally cross-phosphorylate β_C in TF-1 cells. The significance of these crosstalk phenomena is not understood, but the unidirectionality suggests that β_C phosphorylation may contribute to the signaling of cytokines other than GM-CSF, IL-3 and IL-5. Together with the possible involvement of the preformed GM-CSF receptor complex, these observations of receptor crosstalk suggest that β_C may have a role in cytokine receptor signaling, perhaps facilitating certain universal and essential functions such as cellular survival.

Activation of the GM-CSF Family of Receptors: The Intracellular Story

Perhaps the single most striking feature of how cytokines/growth factors stimulate such a diverse range of cellular responses is their comparative redundancy in signaling rather than their specificity. It is becoming increasingly apparent that an extremely broad range of cytokines/growth factors induce a biological response by a common mechanism of receptor dimerization and that activated receptors appear to signal through a very limited number of pathways [28, 41]. The picture of how GM-CSF, IL-3, and IL-5 stimulate cells and the nature of the pathways involved is far from complete; however, these cytokines are known to activate at least three pathways: the JAK/STAT pathway, the ras/MAP kinase pathway, and the PI 3-kinase pathway (Fig. 2). These pathways should not be viewed as being mutually exclusive and may have substantial overlap.

The JAK/STAT Pathway

Although β_C lacks intrinsic kinase activity, GM-CSF, IL-3, and IL-5 have been shown to induce tyrosine phosphorylation of β_C as well as an ever-increasing number of cytoplasmic proteins including: A) kinases, such as PI 3-kinase [42]; B) adaptors, such as Grb2 [43], the insulin receptor substrate-2 (IRS-2) [44], Cbl [45, 46], and SHC [47, 48]; C) guanine nucleotide exchange factors, such as Vav [46, 47, 49]; D) phosphatases, such as SH2-domain protein tyrosine phosphatase-2 ([SHPTP2]; also known as SH2-containing phosphatase-2 or SHP2) [42, 43, 47, 50], and SH2-containing inositol phosphatase (SHIP) [51], and E) transcription factors, such as STAT5 [50, 52]. This phosphorylation is mediated by receptor-associated kinases such as JAK2 [53] and src-family kinases [54], of which JAK2 is better studied and likely to be the most important. The membrane proximal region of β_C contains a conserved proline-rich motif termed "box 1" and serves as a binding site for JAK2 [53]. Following ligand binding and receptor oligomerization, activation of JAK2 kinase activity occurs, most likely due to JAK2 transphosphorylation [53]. JAK2 phosphorylation of β_C and other cytoplasmic signaling proteins is likely to be important in transmitting signals from the cell surface to the nucleus (Fig. 2). The cytoplasmic domain of β_C contains eight tyrosines, six of which are conserved between the mouse and human receptors (Fig. 2: Tyr577, Tyr612, Tyr695, Tyr750, Tyr806, and Tyr866).

Following receptor activation and phosphorylation by either JAK2 or other tyrosine kinases, phosphotyrosine residues within the cytoplasmic domain of β_C could function as high-affinity binding sites for SH2 (src-homology) or

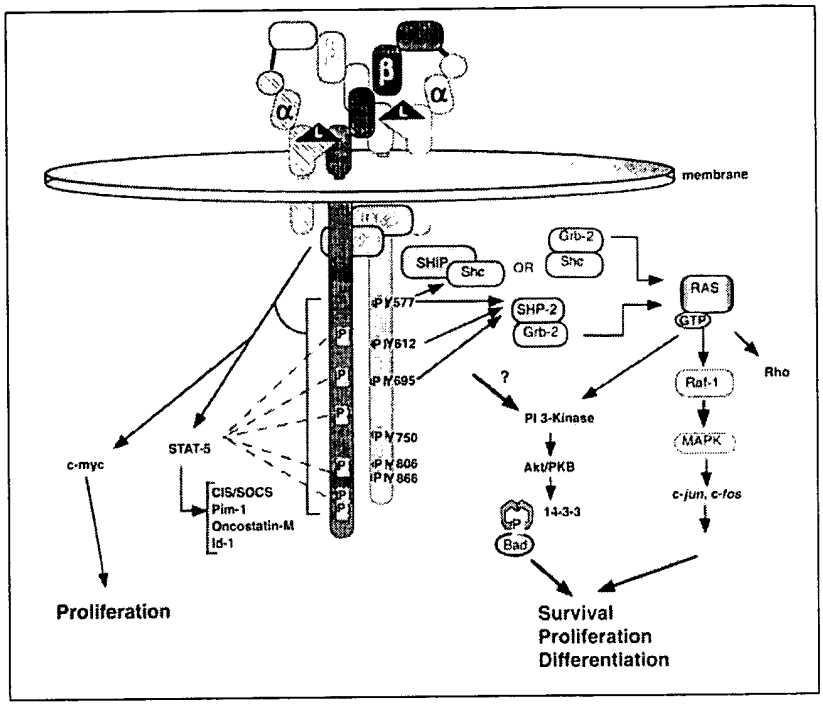

Figure 2. Model showing the signaling pathways stimulated by GM-CSF, IL-3, or IL-5. *Receptor dimerization and activation result in the initiation of a number of signaling cascades which include: A) JAK/STAT pathway and B) the ras/MAP kinase pathway, and the PI 3-kinase pathway. Phosphorylation of tyrosines on β_C (P) allows recruitment and subsequent activation of a variety of SH2 and PTB domain proteins.*

PTB (phosphotyrosine binding) domain proteins [55]. Recruitment of SH2 or PTB domain proteins to the β_C would then serve to couple the activated receptor to downstream signaling pathways [55] (Fig. 2). One such example is the signal transducer and activator of transcription molecule, STAT5. The STAT proteins were originally identified as latent cytoplasmic transcription factors that were activated by JAK proteins in interferon signaling [56]. Although not formally demonstrated for β_C, STAT proteins have been shown to be recruited to tyrosine phosphorylated cytokine receptors via their SH2 domains. Phosphorylation of STAT5 by JAK2 would then result in STAT5 activation, dimerization, and translocation to the nucleus, where it is directly involved in regulating gene transcription [50, 52]. There appears to be a high level of redundancy in terms of which β_C tyrosines are important for STAT5 activation, and all six conserved cytoplasmic tyrosines can mediate STAT5 activation, although to varying extents [43] (Fig. 2). Using dominant-negative STAT5 to abolish STAT5 activity, the JAK/STAT pathway has been shown to be important in the regulation of several GM-CSF-inducible genes, including pim-1, oncostatin M, and Id-1 [57].

Although the functional significance of the induction of these genes is unclear, the JAK/STAT pathway is also important for the induction of cytokine-inducible SH2-containing protein (CIS) [57-59] and the related suppressor of cytokine signaling proteins (SOCS) [60]. These proteins have recently been identified as being involved in a negative feedback pathway which downregulates JAK2 activity following receptor activation [59, 60]. While JAK2 activation has also been found to be essential for c-myc induction, dominant-negative STAT5 did not inhibit this induction, indicating that JAK2 may activate additional, as yet uncharacterized, pathways (Fig. 2).

The ras/MAP Kinase Pathway

Tyrosine mutants of β_C have implicated Tyr577, Tyr612, and Tyr695 in the activation of the ras/mitogen-activated protein (MAP) kinase pathway [48] (Fig. 2). Tyr577 has been shown to interact directly with the PTB domain of the adaptor protein, SHC [43, 61, 62], while tyrosine phosphorylation of Tyr577 and Tyr612 have been shown to be important for the activation of another adaptor protein, grb2 [43]. Other studies have shown that β_C, SHC, and grb2 form a ternary complex [63] which could, in turn, recruit the guanine nucleotide exchange factor, sos, enabling activation of ras and downstream partners of the MAP kinase pathway (Fig. 2). Furthermore, the activation of several components of the ras/MAP kinase pathway, including SHC, ras, Raf, and MAP kinase, can be inhibited by deletion of a domain-encompassing amino acid (a.a.) 626-763, indicating that the tyrosines within this domain (Tyr695 and Tyr750) may also be important in regulating this pathway [48].

The PI 3-Kinase Pathway

PI 3-kinase consists of an 85kDa regulatory subunit containing both SH2 and SH3 domains (p85) and a 110kDa catalytic subunit (p110); it is activated in response to GM-CSF and IL-3 stimulation [48, 64, 65]. Phosphorylation of phosphoinositols by PI 3-kinase generates a class of second messenger molecules such as phosphatidylinositol-3,4-bisphosphate, that in turn regulate the activity of a number of kinases and cytoskeletal proteins [66]. There is no apparent consensus for the direct binding of PI 3-kinase to the β_C;

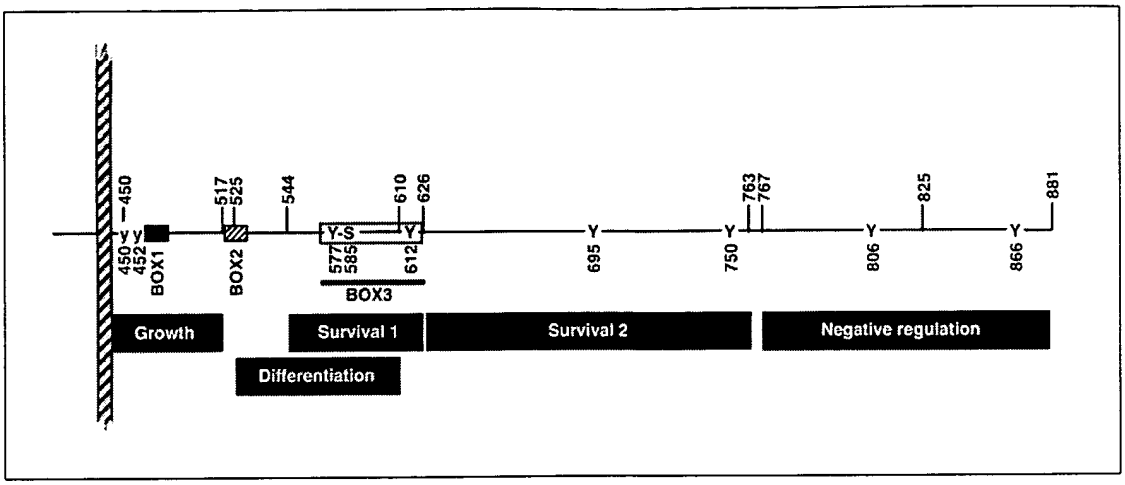

Figure 3. Schematic illustration of the cytoplasmic domains of β_C and their proposed roles in GM-CSF, IL-3, and IL-5 signaling. Truncation mutants of β_C used by a number of laboratories are numbered above the protein [67, 70]. The numbering system of Sakamaki et al. *is used where the N-terminal W-residue following signal peptide cleavage is amino acid number 1, and the mature β_C is expressed as an 881 amino acid protein [67]. Conserved tyrosines (upper-case Y) and nonconserved tyrosines (lower-case Y) are numbered below the protein. Conserved domains boxes 1, 2, and 3 are indicated. Ser585 is within the putative 14-3-3 binding site. The domains found to be important for cell proliferation, differentiation, and survival are indicated by the solid boxes.*

however, recruitment of PI 3-kinase to the GM-CSF and IL-3 receptors may utilize certain adaptor molecules [64] (Fig. 2).

Which Signaling Pathways Emanating from β_C Are Important for Cell Proliferation, Survival, and Differentiation?

Proliferation

Deletion and point mutagenesis of the cytoplasmic domain of β_C have revealed that the pleiotropic activities of GM-CSF, IL-3, and IL-5 are mediated by multiple pathways that are generated from distinct domains (Fig. 3). For instance, the membrane proximal ~35 a.a. is both necessary and sufficient to stimulate a transient mitogenic response in factor-dependent Ba/F3 and CTLL2 cell lines, but this domain alone is unable to support long-term survival [48, 61, 67]. This domain contains a region conserved among type 1 cytokine receptors termed "box 1" and binds JAK2 [53]. Several studies using either box 1 deletions or dominant-negative JAK2 indicate that JAK2 activation is both necessary and sufficient for inducing proliferation [61, 68]. Importantly, JAK2 activation results in the induction of c-myc expression which has been shown to be essential for cell proliferation in a variety of biological settings [48, 68, 69]. The presence of a second conserved motif, termed "box 2" (Fig. 3), ~70 a.a. C-terminal to the transmembrane domain enhances the proliferation signals generated by box 1 but is not absolutely required for either JAK2 activation or proliferation [61].

The results from a number of studies in which individual cytoplasmic tyrosines have been substituted by phenylalanine would suggest that there is a high level of functional redundancy in terms of β_C tyrosine phosphorylation. With the possible exception of a report in which substitution of β_CY750 by phenylalanine resulted in a modest reduction in Ba/F3 cell viability in the presence of serum [47], no single tyrosine has been found to be crucial for mediating any of the known biological activities of GM-CSF, IL-3, or IL-5 [50]. Recent investigations in our laboratory have shown that a mutant β_C in which all eight cytoplasmic tyrosine residues have been substituted by phenylalanine (β_CF8) can mediate FDCP-1 cell proliferation in response to GM-CSF (Fig. 4). Others have shown that β_CF8 can mediate cell proliferation in Ba/F3 cells, although this proliferation is impaired [43, 50]. Despite the importance widely attributed to tyrosine phosphorylation of receptors in initiating signaling, β_C would appear to utilize a mode of signaling that, at least in some cell types, does not require its tyrosine phosphorylation (discussed further below). An additional domain has been mapped, using truncation mutants, to the C-terminal domain of β_C (a.a. 767-881) that negatively regulates proliferative responses to GM-CSF in CTLL cells [70] and the activation of some signaling molecules such as STAT5 [52].

Survival

Distinct from their ability to stimulate cell proliferation, GM-CSF, IL-3, and IL-5 also induce signals important for

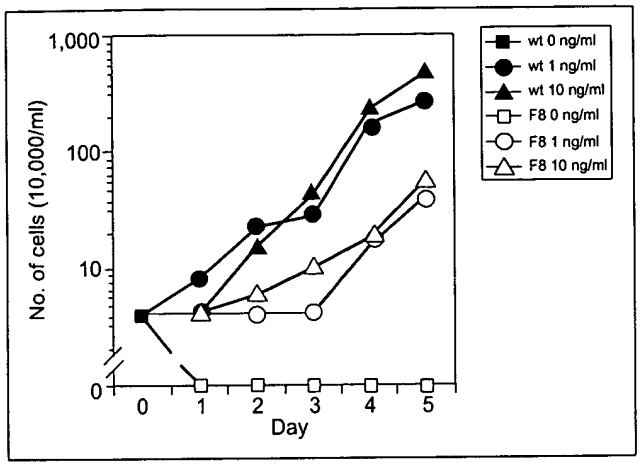

Figure 4. Growth response of myeloid cells expressing wild-type and tyrosine mutant β_c. *Murine FDCP1 cells were stably transfected with human GMRα and either human wild-type β_c or mutant β_cFALL (all eight cytoplasmic tyrosines replaced with phenylalanine). Expression of receptor β_c subunits was comparable as confirmed by FACS analysis. After washing, cells were cultured in human GM-CSF (1 or 10 ng/ml) and cell numbers were determined over five days.*

promoting cell survival. In fact, it is known from experiments performed in cytokine-dependent cells that constitutive proliferation signals without concomitant survival signals result in transient proliferation followed by cell death [71]. For example, cytoplasmic truncations of β_c that preserve box 1 and box 2 allow proliferation but fail to promote long-term survival [47, 71]. Thus, in addition to JAK2 activation, signals originating from membrane-distal regions of the cytoplasmic domain are required for mediating additional biological activities of GM-CSF. To determine the β_c domains important for promoting cell survival, a number of groups have transfected a range of C-terminal β_c truncation mutants into cytokine-dependent Ba/F3 cells [47, 71]. A survival domain that spanned two truncation mutants encompassing a.a. 544-763 was identified [47] (Fig. 3). In the absence of serum, this entire domain (β_c544-763) was required for cell viability (survival domains 1 and 2); however, in the presence of serum, a.a. 544-626 was sufficient for GM-CSF to promote cell survival (survival domain 1) [47].

These results would suggest that: A) there are GM-CSF-mediated survival signals that can, at least in part, be substituted by the presence of serum (β_c626-763), and B) that Ba/F3 cells require a specific GM-CSF-mediated survival signal that cannot be substituted by serum that is mediated by a.a. 544-626 of β_c (Fig. 3, survival domain 1). Indeed, this domain contains a region encompassing a.a. 570 to 626 that we term "box 3," which exhibits unusually high sequence identity between mouse and human β_c (72% identity) when compared with the overall identity of the cytoplasmic domain of β_c (55% identity). The requirement of box 1 and JAK2 activation for the survival response mediated by box 3 is unknown. Although JAK2 activation is required for all known signaling originating from β_c and an essential role seems highly likely, its importance in survival remains to be tested [61, 68].

Identification of a putative survival domain (box 3) raises the question: What motifs within this conserved domain are responsible for signaling, and how do they signal? Although this question addresses one of the most fundamental aspects of GM-CSF biology, the answer has remained elusive. What is apparent, however, is that the survival signals involved are likely to utilize novel pathways and signaling mechanisms. When the possible role of tyrosine phosphorylation in mediating survival signals was examined, some interesting and somewhat surprising results were obtained. Substitution of each of two tyrosines in the box 3 β_c survival domain to phenylalanine (β_cY577F and β_cY612F) had no effect on survival of Ba/F3 cells, although SHC and grb2 activation was reduced for the β_cY577F mutant [72]. Substitution of a tyrosine outside box 3 (β_cY750F) resulted in a modest decrease in GM-CSF-mediated survival in the presence of serum [47].

The dispensability of β_c tyrosine phosphorylation for cell survival is also illustrated by a β_c mutant in which all eight cytoplasmic tyrosines are mutated to phenylalanine (β_cFALL), which is able to support survival in Ba/F3 cells in response to GM-CSF [43, 50]. Clearly, the results obtained with the β_cY577F, β_cY612F, and β_cFALL mutants would suggest that β_c activation invokes novel signals that are important for inducing cell survival that cannot be solely accounted for by receptor tyrosine phosphorylation.

While the presumptive motifs within box 3 of β_c and the mechanism by which it promotes cell survival are unclear, the recent discovery of a novel PI 3-kinase pathway has provided new insights as to how cytokines such as IL-3 promote survival by the prevention of apoptosis. Although PI 3-kinase activity has been well characterized as activating a survival program in response to certain cytokines and growth factors, it was only recently that the downstream signaling partners were identified [66]. IL-3 stimulation of Ba/F3 cells results in the activation of PI 3-kinase and the production of phosphatidylinositols [48, 64]. The binding of phosphatidylinositols to protein kinase B (PKB or Akt) results in activation of its kinase activity [73, 74]. PKB in turn phosphorylates the pro-apoptotic bcl-2 homolog, BAD at Ser136 and Ser112, both of which lie within a suitable context for binding to the 14-3-3 family of adaptor proteins [75, 76].

The binding of 14-3-3 to BAD is thought to sequester the complex to the cytoplasm, thus preventing BAD from exerting its proapoptotic activity (presumably by preventing Bcl-X_L binding on mitochondria) and allowing IL-3-mediated survival [77]. However, in the absence of IL-3 stimulation, the PI

3-kinase/PKB pathway is not activated, and Ser136 of BAD remains unphosphorylated. It is then proposed that nonphosphorylated BAD is no longer sequestered in the cytoplasm by 14-3-3 and translocates to the mitochondria where, in a complex with Bcl-X_L, the events leading to apoptosis are triggered [77]. PI 3-kinase is thought to be recruited to phosphotyrosine residues on activated receptors via its SH2 domain [78]. An interesting question that arises from these studies into IL-3-mediated survival is: How is PI 3-kinase activated by IL-3? Although β_C does not have a tyrosine motif for the recruitment of PI 3-kinase, alternative motifs may exist. Of relevance to this, we have recently obtained evidence that Ser585 in β_C mediates the association of β_C to 14-3-3 upon phosphorylation (unpublished observations). This is potentially an important novel finding because: A) serine phosphorylation of β_C offers an alternative to tyrosine phosphorylation for coupling to specific signaling molecules; B) it has the potential to link β_C via 14-3-3 to signaling molecules that contain a 14-3-3 binding motif, and C) the location of Ser585 is precisely in the β_C region shown to be involved in cell survival and differentiation (box 3, Fig. 3). Biological experiments are currently under way, with β_CS585A mutants to define the functional role of this interaction and the regulation of Ser585 phosphorylation by GM-CSF and IL-3.

Differentiation

The ability of GM-CSF to stimulate differentiation of an M1 murine leukemic cell line expressing a series of deletion β_C mutants has also been examined [70]. M1 cells expressing human GM-CSF receptors differentiate in response to GM-CSF and exhibit many of the morphological and biochemical hallmarks of macrophages. These studies defined a domain encompassing a.a. 525-610 (Fig. 3) that is essential for mediating the ability of GM-CSF to stimulate M1 cell differentiation [70]. It is clear from Figure 3 that the domains responsible for survival and differentiation substantially overlap and also encompass the highly conserved box 3 domain. Thus, one prediction from these findings would be that box 3 contains signaling motifs that are important for mediating survival and differentiation, with the conserved Ser585 as a possible candidate.

Together, the results obtained from studies examining proliferation, survival, and differentiation prompt the question: What is the function of β_C tyrosine phosphorylation in GM-CSF/IL-3/IL-5 receptor activation? One possibility is that within an in vivo context, tyrosine phosphorylation of β_C is important for amplifying, or in some way regulating, GM-CSF/IL-3/IL-5 signals and that such amplification or regulation is difficult to measure in cultured cell lines. Regardless, a number of laboratories have now observed a phosphotyrosine-independent mode of signaling for β_C that allows for proliferation, survival, and differentiation in certain myeloid cells.

Other cytokine receptors have been observed to promote proliferation and differentiation in the absence of tyrosine phosphorylation. Activation of the EPO receptor is essential for the proliferation, survival, and differentiation of erythroid progenitors. Mutant EPO receptors in which all eight cytoplasmic tyrosines were substituted by phenylalanine afforded reduced mitogenesis and β-globin mRNA accumulation compared with wild-type controls [79]. Nevertheless they were able to sustain the growth and survival of the Ba/F3 cells in an EPO-dependent manner.

Biological Roles of GM-CSF/IL-3/IL-5

Numerous in vitro studies documenting the colony-stimulating activity of GM-CSF, IL-3, and IL-5 point toward a role in the production of granulocytes, macrophages, and eosinophils [2]. In addition, GM-CSF, IL-3, and IL-5 are produced by activated T cells and activate the effector function of myeloid cells, implicating these cytokines in the activation of inflammatory and immune responses [1, 2]. It was thus surprising that the most striking phenotype observed following ablation of GM-CSF, IL-3, and IL-5 function by a gene knockout approach was a lung defect [80, 81]. Double-knockout mice obtained by crossing murine β_C -/- mice (which prevents GM-CSF and IL-5-mediated responses but allows IL-3 function via the $\beta_{IL\text{-}3}$ subunit found in mice) with IL-3 -/- mice (which prevents IL-3-meditated responses) were viable and fertile [80]. The β_C/IL-3 double-knockout mice exhibited apparently normal steady-state hemopoiesis with the exception of reduced numbers of eosinophils. However, these mice exhibited a lung defect very similar to a condition observed in humans, termed "pulmonary alveolar proteinosis" (PAP). GM-CSF -/- mice also exhibit PAP, suggesting that lack of GM-CSF signals is responsible for the PAP phenotype in the double-knockout mice [81, 82].

Consistent with the phenotype observed in mice, humans with low levels of β_C expression on peripheral blood cells exhibited PAP with no other apparent hemopoietic disorder [83]. This pathology is characterized by an excessive accumulation of surfactant lipids and proteins in the alveolar spaces. In mice, this accumulation appears to result from a defect in clearance rather than one of overproduction [84]. While numbers of macrophages in GM-CSF -/- lungs appear normal, they are filled with abnormally large amounts of surfactant proteins and lipids [81, 82]. These observations would imply that GM-CSF is important for macrophage function in the lung. The lungs from GM-CSF -/- mice also have increased bacterial and fungal infections with occasional inflammation [82]. Local production of GM-CSF in the lungs of GM-CSF-/- mice using a transgene under the control of a surfactant promoter restores

normal lung function, implying that GM-CSF is likely to function as a classical autocrine/paracrine factor within the lung, a role not previously suspected from in vitro studies [84]. The lungs represent a unique dilemma in biology: How can an organ that allows gas exchange and is in intimate contact with the environment also prevent invasion of a wide range of airborne bacteria, fungus, and other pathogens? While the molecular basis of PAP is unclear, one possibility is that GM-CSF is important for the ability of macrophages in the mammalian lung to phagocytose and degrade surfactant proteins, lipids, and other pathogens in lung alveoli.

Consistent with the proposed role of GM-CSF in inflammation, *Listeria monocytogenes* infection of GM-CSF -/- mice resulted in 50-fold more organisms in the spleen and liver than in wild-type mice [85]. In general, these mice demonstrated reduced long-term survival compared with wild-type controls, with a higher incidence of lung and soft-tissue infections, indicating that GM-CSF expression provides a selective advantage for the survival of the organism [86].

Future Directions/Conclusions

Structure-function studies of GM-CSF, IL-3, IL-5, and their receptors as well as an understanding of the dynamics of receptor dimerization have led to the development of specific receptor antagonists which can block all known receptor functions [23]. By understanding the molecular organization of the cytoplasmic domain of these receptors and by defining discrete regions that regulate either proliferation, survival, differentiation or effector cell function, we envisage that further therapeutic approaches will aim to selectively activate and/or inhibit each function. Therapeutic approaches designed to inhibit cell proliferation while promoting cell differentiation would have clear appeal in pathological conditions such as myeloid leukemia.

The in vitro activity of GM-CSF and IL-3 led to the expectation that these cytokines would be central factors for the production of granulocytes and macrophages in vivo. Recent studies using a gene knockout approach suggest that GM-CSF and IL-3 are dispensable for the steady-state production of these cells in vivo. Although much has been learned using a variety of in vitro systems, the findings in knockout mice highlight the importance of considering function within an in vivo context. Future in vivo approaches for examining some of the unique features of the GM-CSF, IL-3, and IL-5 receptors may rely on the introduction of discrete receptor mutants (knock-ins) on a null receptor background in mice.

The molecular mechanism by which GM-CSF, IL-3, or IL-5 stimulates survival/differentiation remains to be determined, but the possibility that these signals arise from box 3 independently of both proliferation and β_C tyrosine phosphorylation may allow intervention of these pathways with more specific alternatives than phosphotyrosine-based approaches. The ability to increase survival and consequently enhance an inflammatory or immune response to GM-CSF, IL-3, and IL-5 or to decrease GM-CSF autocrine loops associated with leukemia would both be of clinical benefit. Pivotal to such an approach will be the identification of the nature of the survival and differentiative signals.

References

1 Arai KI, Lee F, Miyajima A et al. Cytokines: coordinators of immune and inflammatory responses. Annu Rev Biochem 1990;59:783-836.

2 Nicola NA. Guidebook to Cytokines and Their Receptors. New York: Oxford University Press, Sambrook and Tooze Publications, 1994:171-177.

3 Baldwin GC, Golde DW, Widhopf GF et al. Identification and characterization of a low-affinity granulocyte-macrophage colony-stimulating factor receptor on primary and cultured human melanoma cells. Blood 1991;78:609-615.

4 Baldwin GC, Gasson JC, Kaufman SE et al. Nonhaematopoietic tumor cells express functional GM-CSF receptors. Blood 1989;73:1033-1037.

5 Gearing DP, King JA, Gough NM et al. Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. EMBO J 1989;8:3667-3676.

6 Tavernier J, Devos R, Cornelis S et al. A human high affinity interleukin-5 receptor (IL5r) is composed of an IL5-specific α chain and a β chain shared with the receptor for GM-CSF. Cell 1991;66:1175-1184.

7 Kitamura T, Sato N, Arai K et al. Expression cloning of the human IL-3 receptor cDNA reveals a shared β subunit for the human IL-3 and GM-CSF receptors. Cell 1991;66:1165-1174.

8 Hayashida K, Kitamura T, Gorman DM et al. Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high-affinity GM-CSF receptor. Proc Natl Acad Sci USA 1990;87:9655-9659.

9 Robb L, Drinkwater CC, Metcalf D et al. Hematopoietic and lung abnormalities in mice with a null mutation of the common β subunit of the receptors for granulocyte-macrophage colony-stimulating factor and interleukins 3 and 5. Proc Natl Acad Sci USA 1995;92:9565-9569.

10 Muto A, Watanabe S, Miyajima A et al. High affinity chimeric human granulocyte-macrophage colony-stimulating factor receptor carrying the cytoplasmic domain of the beta subunit but not the alpha subunit transduces growth promoting signals in Ba/F3 cells. Biochem Biophys Res Commun 1995;208:368-375.

11 Bagley CJ, Woodcock JM, Stomski FC et al. The structural and functional basis of cytokine receptor activation: lessons from the common β subunit of the granulocyte-macrophage colony-stimulating factor, interleukin-3 (IL-3) and IL-5 receptors. Blood 1997;89:1471-1482.

12 Hara T, Miyajima A. Two distinct functional high affinity receptors for mouse interleukin-3 (IL-3). EMBO J 1992;11:1875-1884.

13 Nicola NA, Smith A, Robb L et al. The structural basis of the biological actions of the GM-CSF receptor. Ciba Found Symp 1997;204:19-27.

14 Metcalf D, Nicola NA, Gearing DP et al. Low-affinity placenta-derived receptors for human granulocyte-macrophage colony-stimulating factor can deliver a proliferative signal to murine hemopoietic cells. Proc Natl Acad Sci USA 1990;87:4670-4674.

15 Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 1990;87:6934-6938.

16 Lyne PD, Bamborough P, Duncan D et al. Molecular modeling of the GM-CSF and IL-3 receptor complexes. Protein Science 1995;4:2223-2233.

17 De Vos AM, Ultsch M, Kossiakoff AA. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 1992;255:306-312.

18 Hilton DJ, Watowich SS, Katz L et al. Saturation mutagenesis of the WSXWS motif of the erythropoietin receptor. J Biol Chem 1996;271:4699-4708.

19 Baumgartner JW, Wells CA, Chen CM et al. The role of the WSXWS equivalent motif in growth hormone receptor function. J Biol Chem 1994;269:29094-29101.

20 Hercus TR, Cambareri B, Dottore M et al. Identification of residues in the first and fourth helices of human granulocyte-macrophage colony stimulating factor involved in binding to the α- and β- chains of the receptor. Blood 1994;83:3500-3508.

21 Rajotte D, Cadieux C, Haman A et al. Crucial role of the residue Arg280 at the F'-G' loop of the human GM-CSF receptor alpha chain for ligand recognition. J Exp Med 1997;185:1939-1950.

22 Barry SC, Bagley CJ, Phillips J et al. Two contiguous residues in human interleukin-3, Asp^{21} and Glu^{22}, selectively interact with the α- and β-chains of its receptor and participate in function. J Biol Chem 1994;269:8488-8492.

23 Hercus TR, Bagley CJ, Cambareri B et al. Specific human granulocyte-macrophage colony-stimulating factor antagonists. Proc Natl Acad Sci USA 1994;91:5838-5842.

24 Tavernier J, Tuypens T, Verhee A et al. Identification of receptor-binding domains on human interleukin 5 and design of an interleukin 5-derived receptor antagonist. Proc Natl Acad Sci USA 1995;92:5194-5198.

25 Woodcock JM, Zacharakis B, Plaetinck G et al. Three residues in the common β chain of the human GM-CSF, IL-3 and IL-5 receptors are essential for GM-CSF and IL-5 but not IL-3 high affinity binding and interact with Glu^{21} of GM-CSF. EMBO J 1994;13:5176-5185.

26 Lock P, Metcalf D, Nicola NA. Histidine-367 of the human common beta chain of the receptor is critical for high-affinity binding of human granulocyte-macrophage colony-stimulating factor. Proc Natl Acad Sci USA 1994;91:252-256.

27 Woodcock JM, Bagley CJ, Zacharakis B et al. A single tyrosine residue in the membrane proximal domain of the GM-CSF, IL-3 and IL-5 receptor common β chain is necessary and sufficient for high affinity binding and signaling by all three ligands. J Biol Chem 1996;271:25999-26006.

28 Heldin C-H. Dimerization of cell surface receptors in signal transduction. Cell 1995;80:213-223.

29 Sakamaki K, Wang HM, Miyajima I et al. Ligand-dependent activation of chimeric receptors with the cytoplasmic domain of the interleukin-3 receptor beta subunit (beta IL3). J Biol Chem 1993;268:15833-15839.

30 Lia F, Rajotte D, Clark SC et al. A dominant negative granulocyte-macrophage colony-simulating factor receptor alpha chain reveals the multimeric structure of the receptor complex. J Biol Chem 1996;271:28287-28293.

31 Patel N, Herrman JM, Timans JC et al. Functional replacement of cytokine receptor extracellular domains by leucine zippers. J Biol Chem 1996;271:30386-30391.

32 Stahl N, Yancopolous GD. The alphas, betas, and kinases of cytokine complexes. Cell 1993;74:587-590.

33 Stomski FC, Woodcock JM, Zacharakis B et al. Identification of a Cys Motif in the Common β chain of the IL-3, GM-CSF and IL-5 receptors essential for disulfide-linked receptor heterodimerization and activation of all three receptors. J Biol Chem 1998;273:1192-1199.

34 Ronco LV, Silverman SL, Wong SG et al. Identification of conserved amino acids in the human granulocyte-macrophage colony-stimulating factor receptor alpha subunit critical for function. Evidence for formation of a heterodimeric receptor complex prior to ligand binding. J Biol Chem 1994;269:277-283.

35 Woodcock JM, McClure B, Stomski FC et al. The human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor exists as a preformed receptor complex that can be activated by GM-CSF interleukin-3, or interleukin-5. Blood 1997;90:3005-3017.

36 Gamett DC, Pearson G, Cerione RA et al. Secondary dimerization between members of the epidermal growth factor receptor family. J Biol Chem 1997;272:12052-12056.

37 Soltoff SP, Carraway KL, Prigent SA et al. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol Cell Biol 1994;14:3550-3558.

38 Pan CX, Fukunaga R, Yonehara S et al. Unidirectional cross-phosphorylation between the granulocyte colony-stimulating factor and interleukin 3 receptors. J Biol Chem 1993;268:25818-25823.

39 Liu L, Cutler RL, Mui ALF et al. Steel factor stimulates the serine/threonine phosphorylation of the interleukin-3 receptor. J Biol Chem 1994;269:16774-16779.

40 Hanazono Y, Sasaki K, Nitta H et al. Erythropoietin induces tyrosine phosphorylation of the beta chain of the GM-CSF receptor. Biochem Biophys Res Commun 1995;208:1060-1066.

41 Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179-185.

42 Welham MJ, Dechert U, Leslie KB et al. Interleukin (IL)-3 and granulocyte/macrophage colony-stimulating factor, but not IL-4, induce tyrosine phosphorylation, activation, and association of SHPTP2 with Grb2 and phosphatidylinositol 3'-kinase. J Biol Chem 1994;269:23764-23768.

43 Okuda K, Smith L, Griffin JD et al. Signaling functions of the tyrosine residues in the β_c chain of the granulocyte-macrophage colony-stimulating factor receptor. Blood 1997;90:4759-4766.

44 Welham MJ, Bone H, Levings M et al. Insulin receptor substrate-2 is the major 170-kDa protein phosphorylated on tyrosine in response to cytokines in murine lymphohemopoietic cells. J Biol Chem 1997;272:1377-1381.

45 Odai H, Sasaki K, Iwamatsu A et al. The proto-oncogene product c-Cbl becomes tyrosine phosphorylated by stimulation with GM-CSF or Epo and constitutively binds to the SH3 domain of Grb2/Ash in human hematopoietic cells. J Biol Chem 1995;270:10800-10805.

46 Hanazono Y, Odai H, Sasaki K et al. Proto-oncogene products Vav and c-Cbl are involved in the signal transduction through Grb2/Ash in hematopoietic cells. Acta Haematol 1996;95:236-242.

47 Inhorn RC, Carlesso N, Durstin M et al. Identification of a viability domain in the granulocyte/macrophage colony-stimulating factor receptor beta-chain involving tyrosine-750. Proc Natl Acad Sci USA 1995;92:8665-8669.

48 Sato N, Sakamaki K, Terada N et al. Signal transduction by the high affinity GM-CSF receptor: two distinct cytoplasmic regions of the common β subunit responsible for different signaling. EMBO J 1993;12:4181-4189.

49 Matsuguchi T, Inhorn RC, Carlesso N et al. Tyrosine phosphorylation of p95Vav in myeloid cells is regulated by GM-CSF, IL-3 and steel factor and is constitutively increased by p210BCR/ABL. EMBO J 1995;14:257-265.

50 Itoh T, Liu R, Yokota T et al. Definition of the role of tyrosine residues of the common beta subunit regulating multiple signaling pathways of granulocyte-macrophage colony-stimulating factor receptor. Mol Cell Biol 1998;18:742-752.

51 Odai H, Sasaki K, Iwamatsu A et al. Purification and molecular cloning of SH2- and SH3-containing inositol polyphosphate-5-phosphatase, which is involved in the signaling pathway of granulocyte-macrophage colony-stimulating factor, erythropoietin, and Bcr-Abl. Blood 1997;89:2745-2756.

52 Mui AL-F, Wakao H, O'Farrell A-M et al. Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. EMBO J 1995;14:1166-1175.

53 Quelle FW, Sato N, Witthuhn BA et al. JAK2 associates with the β_c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane proximal region. Mol Cell Biol 1994;14:4335-4341.

54 Chaturvedi P, Reddy MV, Reddy EP. Src kinases and not JAKs activate STATs during IL-3 induced myeloid cell proliferation. Oncogene 1998;16:1749-1758.

55 Pawson T, Scott JD. Signaling through scaffold, anchoring, and adaptor proteins. Science 1997;278:2075-2080.

56 Darnell Jr. JE, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264:1415-1421.

57 Mui AL, Wakao H, Kinoshita T et al. Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. EMBO J 1996;15:2425-2433.

58 Yoshimura A, Ohkubo T, Kiguchi T et al. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. EMBO J 1995;14:2816-2826.

59 Endo TA, Masuhara M, Yokouchi M et al. A new protein containing an SH2 domain that inhibits JAK kinases. Nature 1997;387:921-924.

60 Starr R, Willson T, Viney E et al. A family of cytokine inducible inhibitors of signaling. Nature 1997;387:917-921.

61 Itoh T, Muto A, Watanabe S et al. Granulocyte-macrophage colony-stimulating factor provokes RAS activation and transcription of c-fos through different modes of signaling. J Biol Chem 1996;271:7587-7592.

62 Pratt JC, Weiss M, Sieff CA et al. Evidence for a physical association between the Shc-PTB domain and the β_c of the granulocyte macrophage colony-stimulating factor. J Biol Chem 1996;271:12137-12140.

63 Lanfrancone L, Pelicci G, Brizzi M et al. Overexpression of Shc proteins potentiates the proliferative response to granulocyte-macrophage colony-stimulating factor and recruitment of Grb/SoS and Grb2/p140 complexes to the beta receptor subunit. Oncogene 1995;10:907-917.

64 Jucker M, Feldman RA. Identification of a new adaptor protein that may link the common beta subunit of the receptor for GM-CSF, IL-3 and IL-5 to phosphatidylinositol 3-kinase. J Biol Chem 1995;270:27817-27822.

65 Welham MJ, Dechert U, Leslies KB et al. Il-3 and GM-CSF, but not IL-4, induce tyrosine phosphorylation, activation and association of SHPTP2 with Grb2 and PI3-kinase. J Biol Chem 1994;269:23764-23768.

66 Franke TF, Kaplan DR, Cantley LC. PI3K: Downstream AKTion blocks apoptosis. Cell 1997;88:435-437.

67 Sakamaki K, Miyajima I, Kitamura T et al. Critical cytoplasmic domains of the common beta subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation. EMBO J 1992;11:3541-3549.

68 Watanabe S, Itoh T, Arai K. JAK2 is essential for activation of c-fos and c-myc promoters and cell proliferation through the human granulocyte-macrophage colony-stimulating factor receptor in Ba/F3 cells. J Biol Chem 1996;271:12681-12686.

69 Steiner P, Rudolph B, Muller D et al. The functions of Myc in cell cycle progression and apoptosis. Prog Cell Cycle Res 1996;2:73-82.

70 Smith A, Metcalf D, Nicola NA. Cytoplasmic domains of the common β-chain of the GM-CSF/IL-3/IL-5 receptors that are required for inducing differentiation or clonal suppression in myeloid leukaemic cell lines. EMBO J 1997;16:451-464.

71 Kinoshita T, Yokota T, Arai K et al. Suppression of apoptotic death in hematopoietic cells by signaling through the IL-3/GM-CSF receptors. EMBO J 1995;14:266-275.

72 Durstin M, Inhorn RC, Griffin JD. Tyrosine phosphorylation of Shc is not required for proliferation or viability signaling by granulocyte-macrophage colony-stimulating factor in hematopoietic cell lines. J Immunol 1996;157:534-540.

73 Dudek H, Datta SR, Franke TF et al. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 1997;275:661-665.

74 Franke TF, Kaplan DR, Cantley LC et al. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science 1997;275:665-668.

75 del Peso L, Gonzalez-Garcia M, Page C et al. Interleukin-3-induced phosphorylation of Bad through the protein kinase Akt. Science 1997;278:687-689.

76 Yaffe MB, Rittinger K, Volinia S et al. The structural basis for 14-3-3:phosphopeptide binding specificity. Cell 1997;91:961-971.

77 Zha J, Harada H, Yang E et al. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not bcl-x_L. Cell 1996;87:619-628.

78 Duronio V, Scheid MP, Ettinger S. Downstream signaling events regulated by phosphatidylinositol 3-kinase activity. Cell Signal 1998;10:233-239.

79 Damen JE, Liu L, Wakao H et al. The role of erythropoietin receptor tyrosine phosphorylation in erythropoietin-induced proliferation. Leukemia 1997;11:423-425.

80 Nishinakamura R, Miyajima A, Mee PJ et al. Hematopoiesis in mice lacking the entire granulocyte-macrophage colony-stimulating factor/interleukin-3/interleukin-5 functions. Blood 1996;88:2458-2464.

81 Dranoff G, Crawford AD, Sadelain M et al. Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. Science 1994;264:713-716.

82 Stanley E, Lieschke GJ, Grail D et al. Granulocyte-macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. Proc Natl Acad Sci USA 1994;91:5592-5596.

83 Dirksen U, Nishinakamura R, Groneck P et al. Human pulmonary alveolar proteinosis associated with a defect in GM-CSF/IL-3/IL-5 receptor common beta chain expression. J Clin Invest 1997;100:2211-2217.

84 Huffman JA, Hull WM, Dranoff G et al. Pulmonary epithelial cell expression of GM-CSF corrects the alveolar proteinosis in GM-CSF deficient mice. J Clin Invest 1996;97:649-655.

85 Zhan Y, Lieschke GJ, Grail D et al. Essential roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of *listeria monocytogenes*-infected mice. Blood 1998;91:863-869.

86 Seymour JF, Lieschke GJ, Grail D et al. Mice lacking both granulocyte colony-stimulating factor (CSF) and granulocyte-macrophage CSF have impaired reproductive capacity, perturbed neonatal granulopoiesis, lung disease, amyloidosis, and reduced long-term survival. Blood 1997;90:3037-3049.